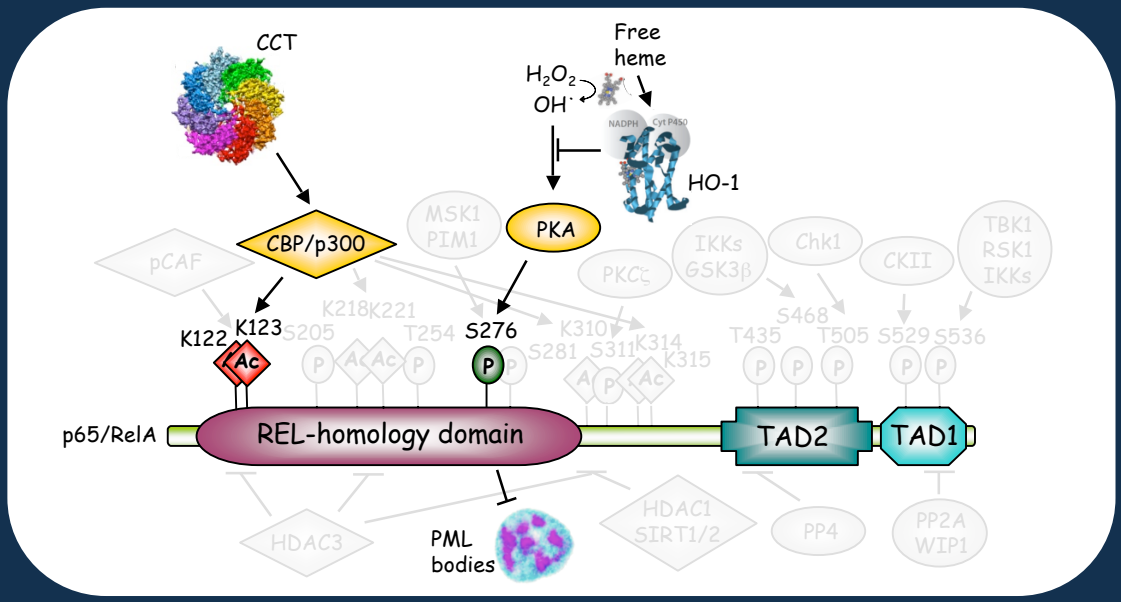
Identification of mechanisms controlling the transcriptional activity of nuclear factor kappa B (NF-κB) p65/RelA

Nadja Pejanovic



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
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Acknowledgments

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Thank You!

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List of Figures and Tables

Glossary

Aq	*Amphimedon queenslandica*
ATP	adenosine triphosphate
BACH	BTB and CNC homologue
BAF	B cell-activating factor
Bcl	B cell CLL/lymphoma
BM	binding mutant
BR	bilirubin
BTB	broad-complex tramtrack and bric a brac
BV	biliverdin
CBP	CREB-binding protein
CCL	chemokine (C-C motif) ligand
CCT	chaperonin containing TCP1
Cdc	cell-division cycle protein
CDK	cyclin-dependent kinase
CNC	cap'and'collar
CO	carbon monoxide
COMM	copper metabolism MURR1
COMMD	COMM-domain-containing protein
COX	cyclooxygenase
Cr	*Carcinoscorpius rotundicauda*
CREB	cyclic AMP-responsive element binding protein
CV	coefficient of variation
CXCL	chemokine (C-X-C motif) ligand
DFO	deferoxamine mesylate
Dif	dorsal-related immunity factor
DN	dominant negative
Dnr	Defense repressor
Drs	*Drosomycin*
dsRNA	double stranded RNA
E1	ubiquitin activating enzyme

E2	ubiquitin conjugating enzyme
E3	ubiquitn protein ligase
EC	endothelial cell
EMSA	electromobility shift assay
FBXL	F-box and leucine-rich repeat protein
Fe	iron
FtH	heavy chain ferritin
GFP	green fluorescent protein
GLP	G9A-like protein
Gprk	G protein-coupled receptor kinase
GRK	G protein-coupled receptor kinase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEK	human embryonic kidney
HKDM	histone lysine demethylases
HKMT	histone lysine methyltransferases
HO	heme oxygenase
HPRT	hypoxanthine guanine phosphoribosyl transferase
IAP	inhibitor of apoptosis protein
ICAM	intercellular adhesion molecule
IKK	IκB kinase
IL	interleukin
iNOS	indusible nitric oxide synthase
IP10	interferon gamma induced protein
IκB	inhibitor of κB
K	lysine
LIM	abnormal cell lineage 11-islet 1-mechanosensory abnormal 3
LPS	lipopolysaccharide
luc	*luciferase*
Luc	firefly
MAMP	microbial associated molecular pattern
MEF	Mouse embryonic fibriblast

MIP macrophage inflammatory protein

Mø macrophage

mRNA messenger RNA

MSK mitogen- and stress-activated kinase

MyD88 myeloid differentiation primary response gene 88

NADPH nicotinamide adenine dinucleotide phosphate reduced

NALP NACHT-leucine-rich-repeat and pyrin-domain-containing protein

NEMO NF-κB essential modulator

NES nuclear export sequence

NF-κB nuclear factor kappa B

NIK NF-κB inducing kinase

NLS nuclear localization sequence

NO nitric oxide

NRF nuclear factor erythroid 2-related factor

NSD nuclear receptor-binding SET domain- containing protein

Nv *Nematostella vectensis*

P/CAF p300/CBP-associated factor

PCD programmed cell death

PDLIM nuclear protein containing PDZ and LIM domains

PDZ postsynaptic density 65-disc large-zonula occludens 1

PG prostaglandin

PK protein kinase

PLK polo-like kinase

PML promyelocitic leukemia

PP2A protein phosphatase 2A

PRR pattern recognition receptor

P-TEF positive transcription elongation factor

qPCR real-time quantitative polymerase chain reaction

Rb Retinoblastoma protein

Ren Renilla

RHD Rel homology domain

RNAi RNA interference

RNS	reactive nitrogen species
ROS	reactive oxygen species
RSK	ribosomal protein S6 kinase
S	serine
sc	scrambled
SET	suppressor of variegation-enhancer of zeste-trithorax
SETD	SET-domain containing protein
shRNA	small hairpin RNA
SIRT	sirtuin
SMRT	silencing mediator of retinoid and thyroid hormone
SOCS	suppressor of cytokine signaling
T	threonine
TAD	transactivation domain
TBK	TANK-binding kinase
Tcp-1	chaperonin containing Tcp-1
TET	tetracycline operon
TF	transcription factor
TGF-β	transforming growth factor beta
TLR	Toll like receptor
TNF	tumor necrosis factor
Toll 10b	constitutively active form of Toll
UV	ultra violet
VCAM	vascular adhesion molecule
VHL	von Hippel-Lin
WIP	wild-type p53-induced phosphatase
wt	wild type
Y	tyrosine
YFP	yellow fluorescent protein

Preface

This Thesis describes the data obtained during the research work performed from October 2005 to February 2011 at the Instituto Gulbenkian de Ciência and Weill Cornell Medical College under the scientific supervision of Miguel Soares, PhD and Josef Anrather, V.M.D.

The Thesis is organized in 5 chapters, which are preceded by a summary written both English and Portuguese. An introductory review on the subject is provided in Chapter I. In Chapters II, III, and IV the original observations obtained during the research period are presented and discussed. Chapter V consists in an extended discussion aiming at integrating the results presented in the previous Chapters.

Summary

Living in an ever-changing environment, cells have evolved numerous mechanisms that allow their assessment of the environment, their communication with each other and the integration of the information in order to adapt to the environmental changes and to maintain their homeostasis. In the presence of harmful stimuli, such as infection or tissue damage, a series of defensive sequential events, including the activation of vascular endothelial cells (EC), are initiated and result in the inflammatory stress response. Much of the inflammatory response is regulated at the level of gene transcription through the expression of a number of proinflammatory genes such as adhesion molecules and stress-responsive cytoprotective genes, e.g. heme oxygenase 1 (HO-1). The nuclear factor kappa B (NF-κB)/Rel family of transcription factors (TF) plays a predominant role in regulating these responses. NF-κB activation is strictly required to trigger the expression of proinflammatory genes associated with inflammation. However, uncontrolled NF-κB activity can result in a failure to resolve inflammation. This in turn may contribute to the establishment of a growing number of pathological conditions. It is therefore imperative to temporally restrict NF-κB activity to target gene subsets needed to execute specific stages of the inflammatory process such as activation, resolution and termination. Among other mechanisms, post-translational modifications, including those exerted directly on Rel proteins, and especially the major transcriptionally active subunit RelA, probably have a key role in the fine-tuning of NF-κB-dependent transcription. While rapidly and reversibly regulating RelA activity by modulating its interaction with cofactors of the transcriptional machinery (i.e. RelA phosphorylation) and RelA DNA binding (i.e. RelA acetylation), post-translational modifications can also

promote RelA translocation to specific subnuclear compartments, through, for example, RelA ubiquitination and phosphorylation, thereby adding an additional layer of control over NF-κB-dependent gene transcription. These distinct functional outcomes determined by specific post-translational modifications have presumably evolved to ensure the specific temporal and spatial transcriptional rate of a subset of RelA-dependent genes.

The aim of this Thesis was to reveal regulatory mechanisms conferring specific outcomes via modulation of RelA post-translational modifications. We have asked whether inflammatory mediators, such as HO-1, dictate the outcome of inflammatory response by inducing and/or inhibiting RelA post-translational modifications. Moreover, we characterized the mechanism regulating gene-restricted transcriptional activity of differentially phosphorylated serine (S) 276 RelA molecules. Finally, we set to identify evolutionary conserved regulators of RelA transcriptional activity. Overall, the findings presented in this Thesis suggest three mechanisms regulating RelA transcriptional activity in a manner that resolves inflammation.

1) RelA phosphorylation by protein kinase A (PKA) at S276 induces RelA conformational changes, promoting its interaction with cofactors of transcriptional machinery, thereby providing a mechanism critical for the expression of a subset of NF-κB-dependent genes. Sustained S276 phosphorylation is associated with an increasing number of inflammatory diseases. We hereby demonstrate that, under oxidative stress, HO-1 inhibits RelA S276 phosphorylation, down-modulating TNF-induced transcription of adhesion molecules associated with the proinflammatory phenotype of activated EC. This anti-inflammatory and cytoprotective effect is mediated via the ability of HO-1 to reduce the level of intracellular non protein-bound iron (labile Fe) and hence the production of reactive oxygen species (ROS), which can

promote NF-κB-dependent transcription via PKA-induced RelA S276 phosphorylation.

2) Phosphorylation of RelA S276 is not globally required. Hypophosphorylated RelA, mimicked by S276A mutation, has similar transcriptional activity on a subset of promoters, compared to wild type RelA. We found that RelA S276A phosphorylation mutant translocates to promyelocitic leukemia (PML) nuclear bodies, an event possibly associated with its gene-specific transcriptional activity. We suggest that RelA S276 phosphorylation prevents this translocation via a mechanism that depends on RelA DNA binding. This occurs presumably via the activation of PKA.

3) Rel proteins and the mechanisms controlling its transcriptional activity are evolutionary conserved. This is also true for post-translational modifications, which can regulate the transcriptional activity of *Drosophila* Rel protein Dorsal. We identified chaperonin containing TCP1 (CCT) as a regulator of transcriptional activity of *Drosophila* Rel proteins, i.e. Dorsal and Dorsal-related immunity factor (Dif), using a RNA interference (RNAi) screening approach. The CCT also regulated NF-κB transcriptional activity in mammalian cells, acting in a promoter-specific context. While promoting NF-κB-dependent transcription during the early phase of RelA activation, CCT diminished gene expression during the late phase of RelA transcriptional activity. The later effect was associated with decreased RelA DNA binding and termination of NF-κB activity via a mechanism involving the modulation of RelA lysine (K) 122 and K123 acetylation, presumably by altering the activity of histone acetyl transferase CREB-binding protein (CBP).

Sumário

Por viverem num ambiente em constante mudança, as células desenvolveram numerosos mecanismos que lhes permitem detetar mudanças no ambiente, comunicar essas mudanças entre si, bem como integrar estas informações de maneira a adaptarem-se a tais mudanças, mantendo a sua homeostase. Na presença de estímulos nocivos, como infeções ou danos teciduais, uma série de eventos sequenciais, incluindo a ativação de células endoteliais (CE), são iniciados, com o objetivo de defender o organismo, culminando na resposta inflamatória. Grande parte da resposta inflamatória é regulada ao nível transcricional, através da expressão de vários genes pró-inflamatórios, tais como moléculas de adesão, mas também de genes com propriedades citoprotetoras, como por exemplo a enzima heme oxigenase-1 (HO-1). A família de fatores de transcrição (FT) factor nuclear kappa B (NF-κB)/Rel tem um papel fundamental na regulação da resposta inflamatória. A ativação do NF-κB é essencial para a expressão de genes pró-inflamatórios. No entanto, a ativação descontrolada deste FT pode impedir a resolução do processo inflamatório, levando ao desenvolvimento de uma grande variedade de patologias. Desta forma, é imperativo que a atividade do NF-κB seja controlada de maneira a permitir que os conjuntos de genes envolvidos nos diferentes estágios do processo inflamatório, ativação, resolução e término, sejam expressos nas fases adequadas. Um dos principais mecanismos envolvidos na regulação da transcrição, mediada pelo NF-κB, são as modificações pós-traducionais, incluindo as que ocorrem diretamente nas proteínas Rel, e mais especificamente na subunidade com maior atividade transcricional, o RelA. Diferentes modificações pós-traducionais do RelA afetam de maneira diferente a sua atividade. A

fosforilação do RelA pode afetar a sua interação com co-fatores da maquinaria transcricional, enquanto a acetilação afeta a ligação do RelA ao DNA. Para além disso, a ubiquitinação e fosforilação do RelA podem induzir a sua translocação para compartimentos subnucleares, assim adicionando mais uma camada de controlo na transcrição de genes dependents do NF-κB. Possivelmente, todos estes mecanismos evoluíram de maneira a assegurar a transcrição de conjuntos de genes específicos adequados às diversas fases do processo inflamatório.

Esta Tese tem por objetivo revelar a especificidade dos mecanismos reguladores capazes de alterar a atividade do RelA, via modificações pós-traducionais. Concretamente, estudamos se mediadores inflamatórios, como a HO-1, são capazes de alterar o processo inflamatório através da indução de modificações pós-traducionais no RelA. Além disso, identificamos que a fosforilação da serina (S) 276 do RelA é responsável pela modificação da atividade desta molécula. Finalmente, realizamos experiências visando identificar moléculas conservadas evolutivamente, envolvidas na regulação da atividade transcricional do RelA. Os resultados apresentados nesta Tese sugerem três mecanismos reguladores da atividade transcricional do RelA envolvidos na resolução da inflamação.

1) A fosforilação da S276 do RelA pela proteína cinase A (PCA) induz modificações conformacionais que promovem a interação do RelA com co-fatores da maquinaria transcricional. Esse mecanismo induz a expressão de uma fração de genes dependentes do NF-κB. A fosforilação continuada da S276 está associada a uma série de doenças inflamatórias. Neste estudo, demonstramos que em condições de stresse oxidativo, a HO-1 inibe a fosforilação da S276 do RelA, dessa forma inibindo a transcrição de moléculas de adesão, induzida por tratamento com TNF, em células endoteliais. Este efeito anti-inflamatório e citoprotetor, é mediado pela capacidade da HO-1 de reduzir os níveis

intracelulares de ferro (Fe) não ligado a proteínas (Fe lábil). A diminuição dos níveis intracelulares de Fe lábil reduzem o nível de espécies reativas de oxigénio (ROS), que por sua vez, atuam de maneira a promover a transcrição de genes dependentes de NF-κB, via fosforilação da S276 pela PCA.

2) A mutação da S276A no RelA, recriando um RelA hipo-fosforilado, tem uma atividade transcricional semelhante à do RelA do tipo selvagem, numa fração de genes dependentes do NF-κB. Os nossos resultados demonstram que o RelA S276A mutante transloca-se para o núcleo e localiza-se nos corpos nucleares da leucemia promielocítica, um evento possivelmente associado com o fato da sua atividade transcricional ser específica, ou seja, dependente do gene em questão. Os nossos resultados sugerem que a fosforilação da S276 no RelA impede a sua translocação através de um mecanismo dependende da ligação do RelA ao DNA. Possivelmente, este efeito ocorre através da ativação da PCA.

3) As proteínas Rel e os mecanismos que controlam a sua atividade transcricional são conservados evolutivamente. Isto também se aplica para modificações pós-traducionais, uma vez que este mecanismo regula a atividade transcricional da proteína Dorsal, equivalente de RelA em *Drosophila*. Utilizando um rastreio baseado em RNA de interferência, identificamos a chaperonina contendo TCP1 (CCT) como um regulador da atividade transcricional das proteínas Rel presentes em *Drosophila*, Dorsal e factor de imunidade relacionado com o Dorsal (Dif). A CCT também regula a atividade transcricional do NF-κB em células de mamíferos, atuando de forma específica no contexto de cada promotor. A CCT promove a transcrição de genes dependentes do NF-κB na fase inicial da ativação do RelA. No entanto, diminui a expressão destes genes na fase final da ativação. Este último efeito

está associado a uma diminuição da ligação do RelA ao DNA e ao fim da atividade do NF-κB através de um mecanismo que envolve a acetilação das lisinas (K) 122 e K123 do RelA, possivelmente pela alteração da atividade da acetiltransferase das histonas, a proteína de ligação a CREB (CBP).

CHAPTER I
GENERAL INTRODUCTION

I. Prologue

"*When we consider the extreme instability of our bodily structure, its readiness for disturbance by the slightest application of external forces and the rapid onset of its decomposition as soon as favoring circumstances are withdrawn, its persistence through many decades seems almost miraculous. The wonder increases when we realize that the system is open, engaging in free exchange with the outer world, and that the structure itself is not permanent but is being continuously broken down by the wear and tear of action, and as continuously built up again by processes of repair."*
- Walter Bradford Cannon - *"The wisdom of the body"*

Claude Bernard first recognized the significance of the body's *"milieu intérieur"* (internal environment), which was later defined by Walter Bradford Cannon as "*homeostasis*" (from Greek: *ομοιος (hómoios)*, "same" and *στασις (stásis)*, "standing"), i.e. "remaining the same" (1). Homeostasis is a fundamental principle of all living organisms and if one considers "same" as "self", homeostasis can be defined as an ability of the organism to "remain self". A variety of strategies have therefore evolved from unicellular organisms to humans recognizing "self" from "non-self". The outcome of this so called "self-non-self" discrimination can vary from establishing a life long symbiotic relationship to the destruction and elimination of "non-self", as it occurs for commensal microbiota and pathogens, respectively.

In bacteria, "self-non-self" discrimination relies on the expression of various specialized proteins that include antimicrobial peptides activity such as microcins that interfere with the cell cycle of "non-self" microorganisms or enzymes, i.e. restriction endonucleases, recognizing

and eliminating nucleic acids sequences specific to “non-self” (2). As for other unicellular organisms, bacteria developed mechanisms that assure not only maintenance of their individual homeostasis but also that of entire bacterial populations, thus manifesting the first traits of multi-cellular behavior, as seen in eukaryotes. Bacteria have also developed mechanism eliminating “altered self” and eventually “self”, inducing programmed cell death (PCD) (3). Examples include phage-induced PCD, an effect that prevents phage, i.e. “non-self”, growth (4), DNA damage-induced PCD, maintaining the genetic continuity of population by eliminating “altered self” (5) and PCD of a part of bacterial population, i.e. “self”, providing nutrients for the surviving bacterial population (6). These mechanisms described above rely in most cases on newly gene transcription and protein synthesis. Gene transcription in bacteria is mostly turned on by default and is negatively regulated by the presence of specific repressor proteins (7). This mechanism of gene transcription allows bacteria to rapidly adapt to changes in the environment.

The emergence of more complex multi-cellular eukaryotes obliged new mechanism of “self-non-self” discrimination, supplementing those first developed by unicellular prokaryotes. These include the emergence of specialized receptors recognizing “non-self”, “altered-self” as well as “self”, triggering a series of coordinated adaptive events eventually leading to the elimination of inciting stimuli and the return to homeostasis, responses collectively referred to as stress responses (8). Depending on the nature and the extent of stimuli sensed by a given receptor, appropriate stress responses are induced, which can occur at the cellular level, tissue level, referred to as parainflammation, or systemically, referred to as inflammatory response (8, 9).

Inflammation has been known to humans for almost two thousand years, initially as “*rubor et tumor cum calore et dolore*“ (redness and swelling with heat and pain) and later on as “*functio laesa*”

(disturbance of function) and will be briefly described hereby to illustrate the diversity of mechanisms that evolved in order to maintain the homeostasis of complex multi-cellular organisms (see Figure 1.1.). The initiation of inflammatory response relies on the recognition of microbial

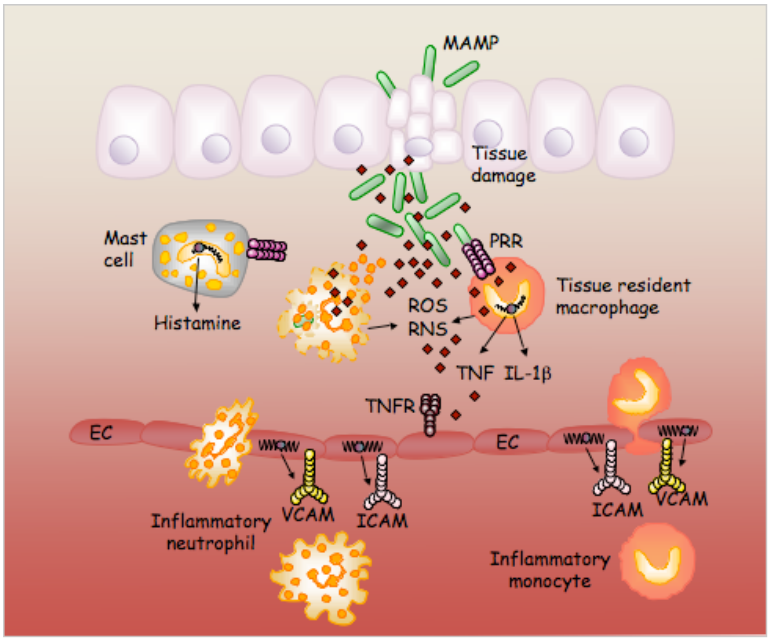

Figure 1.1. The inflammatory response. The initiation of inflammatory response relies on the recognition of microbial associated molecular patterns (MAMP) as well as non microbial molecules by different sensors including pattern recognition receptors (PRR), expressed on tissue resident macrophages and in some tissues by mast cells, triggering a series of signal transduction pathways leading to the expression of inflammatory mediators, i.e. TNF, IL-1β, histamine. These can target vascular endothelial cells (EC), which express several adhesion molecules, i.e. VCAM, ICAM, that play a key role in recruiting circulating leukocytes, i.e. neutrophils and monocytes to the site of inflammation, leading to elimination of the noxious agent upon phagocytosis or via the release of a various molecules with the microbicidial activity, e.g. ROS, RNS.

associated molecular patterns (MAMPs) as well as non-microbial molecules that reveal the presence of “non-self” or “altered self” and as such alert for possible deviations from homeostasis. These molecules, also known as inducers of inflammation, are recognized by specialized sensors, which include evolutionary conserved germline encoded pattern recognition receptors (PRRs), such as toll like receptors (TLRs), recognizing MAMPs as well as other less well characterized sensors recognizing directly or indirectly distinct effects of the inducers on host tissues. The examples include but are not restricted to NACHT-leucine-rich-repeat- and pyrin-domain-containing protein (NALP3) inflammasome recognizing efflux of potassium $(K)^+$ ions, resulting from pore formation during Gram-positive bacterial infection (10) and purinoceptors recognizing adenosine triphosphate (ATP) (11). Engagement of PRR triggers a series of signal transduction pathways leading to the activation of several transcription factors (TF). These can act in a concerted manner to regulate the expression of genes that include mostly a series of inflammatory mediators, such as cytokines, chemokynes and lipid mediators (see Table 1.1.). Inflammatory mediators are recognized by specific receptors expressed by different cell types such as vascular endothelial cells, which play a key role in providing adaptation of blood flow as well as recruiting circulating leukocytes (e.g. neutrophils and monocytes), acting as the effector components of inflammation. As such, the inducers, e.g. MAMPs, sensors, e.g. PRR, mediators and effectors define different types of inflammatory responses aimed at reestablishing homeostasis following its disruption by the presence of toxic molecules associated with the inflammation (8).

In the case of microbial infections, probably the most common cause of inflammation, circulating neutrophils and monocyte/ macrophages (Mø) provide the first line of defense aimed at pathogen killing (see Figure 1.1.). A critical mechanism relies on the release of

INFLAMMATORY MEDIATORS	NF-κB DEPENDENT	REGULATING NF-κB
Vasoactive amines		
histamine		(12)
serotonin		
Vasoactive peptides		
substance P		(13)
kinins		(14)
Complement fragments		
C3a		(15)
C5a		(15)
Lipid mediators		
prostaglandins	*(16)	(17)
leukotrienes		(18)
Cytokines		
tumor necrosis factor (TNF)	(19, 20)	(21, 22)
interleukin-1 (IL-1)	(23)	(21)
IL-6	(24)	(25)
Chemokines		
chemokine (C-C motif) ligand 2 (CCL2)	(26)	(27)
CCL3	(28, 29)	
IL8	(30)	(31)
Proteolytic enzymes		
elastase		(32)
cathepsins	(33)	
matrix metalloproteinases	(34)	
Prothrombotic peptides		
tissue factor	(35)	
Gases		
nitric oxide (NO)	*(36, 37)	(38)
Adhesion molecules		
endothelial (E) selectin	(39, 40)	
platelet (P) selectin	(41)	
intercellular adhesion molecule 1 (ICAM-1)	(42)	
vascular adhesion molecule 1 (VCAM-1)	(43)	

Table 1.1. The inflammatory mediators. Examples of mediators that coordinate the initiation of inflammation. The expression of several of these mediators is induced via the activation of NF-κB and/or can regulate NF-κB activity. *The generation of prostaglandins and NO is indirectly dependent on NF-κB activity via the inducton of cyclooxygenase-2 (COX-2), and nitrix oxide synthase (NOS).

antimicrobial cytotoxic molecules from secretory vesicles called granules, a process referred to as degranulation. The microbicidial activity of activated neutrophils as well as that of Mø is further enhanced via the activation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) phagocyte oxidase complex, generating high levels of superoxide and triggering the formation of other highly toxic reactive oxygen species (ROS) and reactive nitrogen species (RNS) aimed at eliminating the invading pathogen. Given the broad cytotoxic effect of ROS and RNS, their production must be tightly regulated in order to avoid unfettered tissue damage.

Inflammatory responses must be terminated as soon as pathogen clearance has been achieved, as to restore homeostasis, a highly regulated process involving the expression of a variety of stress-responsive genes (see Figure 1.2.) (44). Many of these genes exert their effect directly on vascular endothelial cells inhibiting signal transduction pathways preceding vascular changes, leukocyte infiltration and activation. For example, the enzyme cyclooxygenase-2 (COX-2) produces 15-deoxy-Δ12,14-prostaglandin (PG) J_2, a lipid mediator that restrains the expression of proinflammatory genes in many cells, including intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) in endothelial cells (45), or inducible nitric oxide synthase (iNOS) in Mø (46, 47). Concomitantly, the production of several pro-inflammatory mediators during the initial phase of inflammation is switched towards the production of anti-inflammatory molecules, promoting the resolution of inflammation. This is well illustrated by the transition from the generation of proinflammatory lipid-mediators such as leukotrienes to anti-inflammatory lipoxins, inhibiting further the recruitment of circulating neutrophils into the site of infection (48). At the same time, activated neutrophils involved in pathogen clearance start undergoing PCD, a regulated mechanism limiting tissue

damage caused by the local production of ROS. Neutrophil PCD is associated with the expression of cell surface phosphatidylserine, recognized specifically by receptors expressed by infiltrating Mø (49, 50). This triggers a genetic program in Mø characterized by the

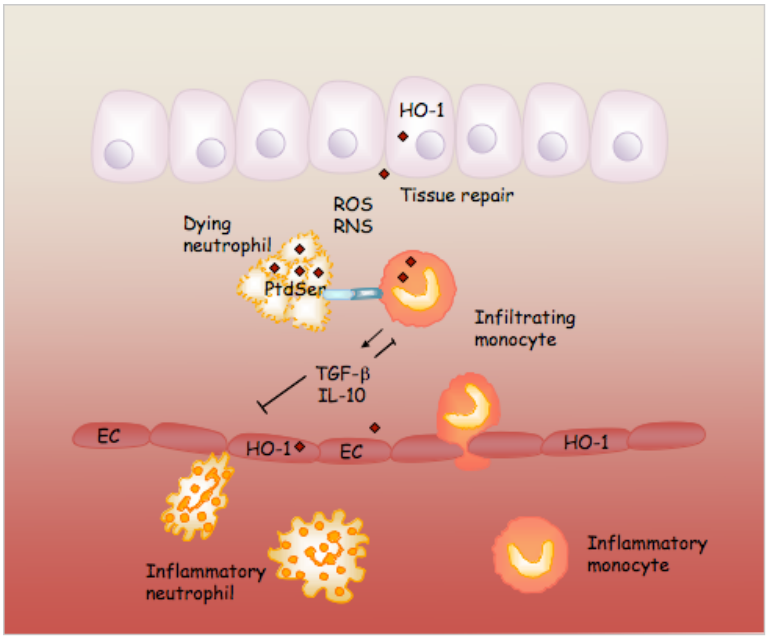

Figure 1.2. The resolution of inflammation. The inflammatory responses must be terminated after pathogen clearance. The expression of several anti-inflammatory mediators inhibits signal transduction pathways preceding vascular changes, leukocyte infiltration and activation. Activated neutrophils at the site of inflammation undergo PCD, limiting tissue damage caused by ROS and RNS production. Expression of phosphatidylserine (PtdSer) results in neutrophil recognition and phagocytosis by infiltrating monocytes, which triggers the production of immunomodulatory molecules such as IL-10 and TGF-β helping resolution of inflammation. At the same time, pro-oxidant environment (ROS and RNS) induces the expression of so-called stress-responsive genes, such as heme oxygenase 1 (HO-1), contributing to the termination of inflammation.

production of immunomodulatory molecules, e.g. transforming growth factor beta (TGF-β), interleukin 10 (IL-10), that limit the proinflammatory phenotype and hence cytotoxic effect associated with the onset of inflammatory reactions (51). Production of free radicals, i.e. ROS and RNS, aimed at destruction of pathogens also induces the expression of genetic programs characterized by the expression of cytoprotective stress-responsive genes such as heme oxygenase-1 (HO-1), contributing further to the termination of inflammation (see Chapter IV).

Expression of HO-1 appears to be critically involved in the return of homeostasis associated with the resolution of inflammatory responses. HO-1 is a cytoprotective enzyme that catalyzes the cleavage of protoporphyrin IX ring of heme into labile iron (Fe), carbon monoxide (CO) and biliverdin (BV), the latest being converted by a biliverdin reductase into the anti-oxidant bilirubin (see Figure 1.3.) (52, 53). Labile Fe, on the other hand, induces heavy chain ferritin (FtH) expression, which mediates cytoprotective effects under a variety of conditions (54-56). CO, while originally thought to be a toxic by-product of heme catabolism, can act as a signalling mediator with anti-inflammatory, cytoprotective and vasodilator properties (57, 58).

The salutary effects of HO-1 appear to be driven by its capacity to limit the deleterious effect of heme. Under homeostasis, heme exists as a prosthetic group of hemoproteins that are essential for various cellular functions including the oxygen transport, i.e. hemoglobin, and storage, i.e. myoglobin (59), generation of cellular energy, i.e. redox cytochromes (60), antioxidant defences, i.e. catalase and production of superoxide anions, i.e. NADPH oxidase, or nitric oxide (NO), i.e. NOS (61, 62). Under oxidative stress, however, some of these hemoproteins are rapidly oxidized, releasing and augmenting the concentrations of non-protein bound (free) heme. This abundant source of Fe is highly deleterious to cells as it can participate in the Fenton reaction, catalyzing

the production of free radicals thus amplifying the cytotoxic effect of ROS (63, 64). Besides this, free heme can be recognized by Mø and neutrophils activating their proinflammatory phenotype, i.e. cytokine expression and ROS production (65-67).

The critical role of HO-1 in the regulation of homeostasis is suggested by the observation that HO-1 deficiency leads to chronic inflammation, characterized by increased number of blood leukocytes, splenomegaly, accumulation of monocyte/Mø in tissues, vascular injury,

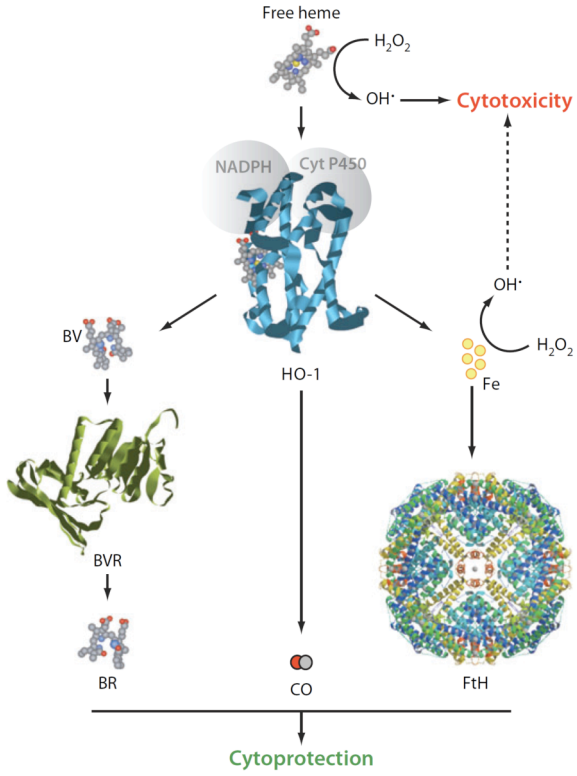

Figure 1.3. The HO-1 system. The cleavage of protoporphyrin IX ring of free heme produces biliverdin (BV), which is converted by biliverdin reductase (BVR) into the bilirubin (BR), CO and labile iron (Fe) that is stored by the ferritin H chain (FtH). Image from (68).

cell death and Fe depositions in tissues and renal damage (69-71). Moreover HO-1 deficiency and heme-mediated oxidative damage have been associated with a growing number of inflammatory-mediated diseases (72). While limiting the accumulation of free heme should *per se* contribute to the salutary effects of HO-1, the products of heme degradation, i.e. Fe, CO and BV, also contribute critically to the salutary effects of HO-1 (68). As demonstrated under Chapter IV, this occurs in part via the regulation of specific TFs regulating the expression of proinflammatory genes, such as the nuclear factor kappa B (NF-κB) family of TF (73).

Tissue repair response is another crucial step in restoring homeostasis in the context of inflammatory responses. Injured tissues are repaired by regeneration of parenchymal cells (the main specialized cells of the tissue or the organ) or, in the event when regeneration is not capable *per se* to restore homeostasis, by connective tissue repair in which scar tissue, e.g. fibroblasts, is substituted for the specialized parenchymal cells of the injured tissue. Numerous chemical mediators and growth factors orchestrate this healing process acting as chemoattractants or promoting cell proliferation. Among many others, chaperonin containing TCP-1 (CCT) have a critical role in sustaining cell proliferation, cytoskeletal proteins polymerization and have been shown to regulate fibroblast motility and contractility (74, 75). As such, CCT will be briefly introduced in the Section II.3.1.

Alike in prokaryotes, many of the responses described above are regulated at the level of transcription, a process that relies on a group of proteins known as TFs (76). Comparative genome analysis suggest that increase in gene and TF number cannot account *per se* for all the diversity of mechanisms maintaining homeostasis in multi-cellular eukaryotes (77). Rather, the complexity of mechanisms regulating gene transcription are accountable for the specific response to a given

environmental change (77). Unicellular eukaryotes in principal have short regulatory DNA sequences located immediately upstream of the coding sequence of their genes that can interact with one or two TFs, driving their transcription. Higher organisms, on the other hand, contain highly structured regulatory sequences scattered both upstream and downstream of the gene coding sequence as well as within introns and often require multiple TFs to regulate gene transcription. This combinatorial mechanism implies not only the coordinated binding of multiple TFs but also their interaction with co-regulators of transcriptional machinery (78). On the other hand, a single TF can drive the expression of hundreds of putative target genes in a cell- and stimulus-specific context, an effect dependent on post-translational regulation of TF (see Figure 1.4.). As eukaryotic transcription and translation are spatially isolated, a first layer of regulation is provided by the TF sequestration in the cytoplasm, requiring their nuclear translocation. While some TFs are newly synthesized in response to a specific stimulus, many are constitutively expressed and retained inactive in the cytoplasm often via the interaction with specific cytoplasmic inhibitory proteins or in the form of inactive precursors (see Section II.2.2). These can be rapidly activated upon stimulus-induced liberation from their respective inhibitors or through post-translational modifications, allowing the active form of the TF to translocate into the nucleus and bind to specific DNA binding sequences in the *cis*-regulatory DNA region of target genes, eliciting rapid and controlled responses to environmental changes. TFs act often as homo- or hetero-dimeric complexes, further increasing the complexity of possible regulatory mechanisms (see Section II.2.1.). Other than regulation of nuclear translocation, dimerization and DNA binding, TF can contain transactivation domains (TAD) that interact with different transcriptional co-regulators, as well as with chromatin remodeling complexes (see Section II.2.4.). Thus, TF lacking TAD can act as a

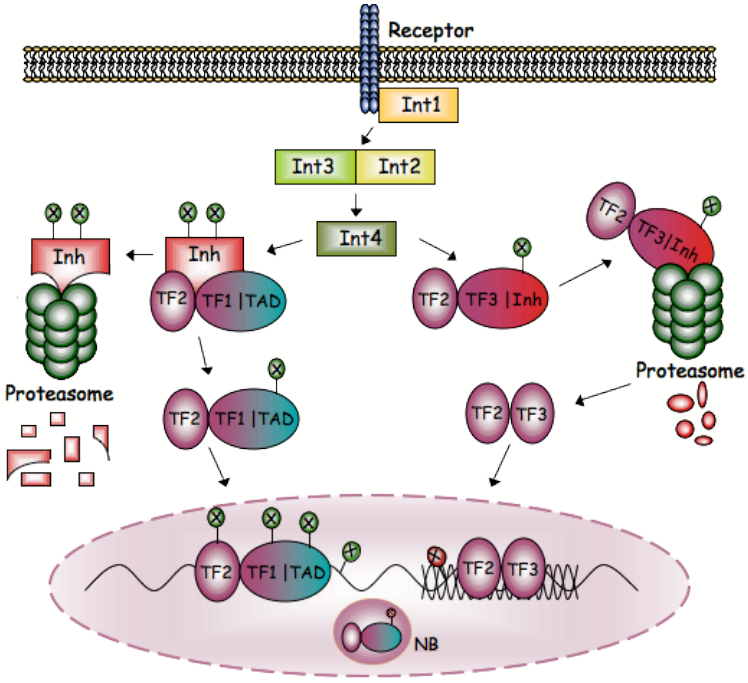

Figure 1.4. Transcriptional regulation. Transciption factors (TF) can be ubiquitously expressed and retained inactive in the cytoplasm via the interaction with specific inhibitory proteins (Inh) or in the form of inactive precursors (TF3 I Inh). Engagement of different receptors can trigger signal transduction pathways (Int1-4), which liberate TF from their respective Inh through post-translational modifications (Inh degradation or TF3 I Inh processing), allowing for TF nuclear translocation. Other than regulation of nuclear translocation, dimerization (TF1-TF2 and TF2-TF3) and DNA binding, TF can contain transactivation domains (TF1 I TAD), defining those transcriptionally active. On the other hand, TF dimer lacking TAD acts as a repressor of transcription (TF2 I TF3). Besides inducing Inh degradation, post-translational modifications can also target TF as well as histones within promoters of target genes, fine-tuning transcriptional response (positively in green or negatively in red) via a mechanism involving TF nuclear translocation, interaction with other proteins, DNA binding, stability, translocation to specific subnuclear compartments so called nuclear bodies (NB) and chromatin remodeling.

repressor of transcription. Post-translational modifications are increasingly recognized as a key mechanism regulating transcriptional activity, acting often as a pre-requisite for TF nuclear translocation, interaction with other proteins, DNA binding and stability (see Section II.2.3.). Moreover, post-translational modifications have been recently implicated in TF translocation into different subnuclear compartments collectively known as nuclear bodies (see Section II.2.5.), which have an important role in regulating gene transcription. While regulation of TF activity is probably the best studied mechanism controlling gene expression there are several other layers of regulation that include: i) chromatin modifications, ii) transcriptional co-factor localization, modifications and degradation, iii) messenger (m) RNA splicing, polyadenylation and localization and iv) micro RNA.

Among the many TF involved in the regulation of inflammatory responses, the pleiotropic TF NF-κB appears to play a central role in these processes. Many of the inducers of inflammation including MAMPs recognized by PRR activate NF-κB. These include molecules associated with bacterial and viral infection, inflammatory cytokines produced in response to microbial recognition, physical and oxidative stress, e.g. ultra violet (UV)-radiation and reactive oxygen intermediates, respectively, resulting in the production inflammatory mediators such as proinflammatory cytokines, chemokines, cell adhesion molecules, immunoreceptors, stress responses genes, cytoprotective genes and growth factors, arguing further for the involvement of NF-κB in the regulation of inflammation (79, 80).

The physiological relevance of NF-κB in the regulation of inflammation has been clarified by loss of function studies in model organisms lacking specific core components of the NF-κB pathway (81-83). Despite the considerable knowledge that these studies have

provided, the detailed aspects of the complexity of NF-κB regulation during the inflammatory responses is only starting to emerge. While NF-κB is essential for the initiation of inflammatory response, e.g. transcription of various proinflammatory mediators (see Table 1.1.), tight regulation and proper termination of NF-κB-dependent transcriptional response is also necessary for the resolution of inflammation and return to homeostasis, e.g. switch from proinflammatory to anti-inflammatory gene transcription, neutrophil PCD. Uncontrolled NF-κB activity has been implicated in the pathogenesis of immune-mediated inflammatory diseases, including some acting as major causes of morbidity and mortality worldwide such as rheumatoid arthritis, asthma and inflammatory bowel disease (84). Moreover, deregulated NF-κB activity has been also associated with cancer development. Presumably, this is related to the fact that persistent NF-κB activity can lead to sustained tissue damage, inflammatory cell infiltration and production of cytokines, chemokines, free radicals and growth factors, which together with the anti-apoptotic effect of the NF-κB promote DNA damage, mutagenesis, cell proliferation and eventually tumor formation (85). As such, regulation of NF-κB activity has been in focus of research in the past twenty-five years and will be the focus of this Thesis.

II. The NF-κB family of transcription factors

The NF-κB signaling system comprises a family of Rel homology domain (RHD)-containing TF (hence the name Rel proteins) and their respective inhibitors, referred to as inhibitors of κB (IκBs) (86). While originally discovered as a nuclear factor binding selectively to the immunoglobulin κ enhancer in B-cells (87), NF-κB family members were later found to be ubiquitously expressed as well as highly conserved throughout evolution.

To date five Rel proteins have been identified in mammals, namely p65/RelA, RelB, cRel, p105/p50 and p100/p52, that exist as homo- or hetero-dimeric complexes (see Figure 1.5.). The dimer RelA-p50, the first identified, is expressed in the majority of cell types (88). RelA, RelB and c-Rel contain a TAD within their C-terminal domain, conferring them with intrinsic transcriptional activity. Since NF-κBs are constitutively expressed in most cells, they can respond rapidly to a variety of triggers and multiple layers of control have evolved ensuring the tight regulation of their transcriptional activity (see Section II.2.). The central mechanism

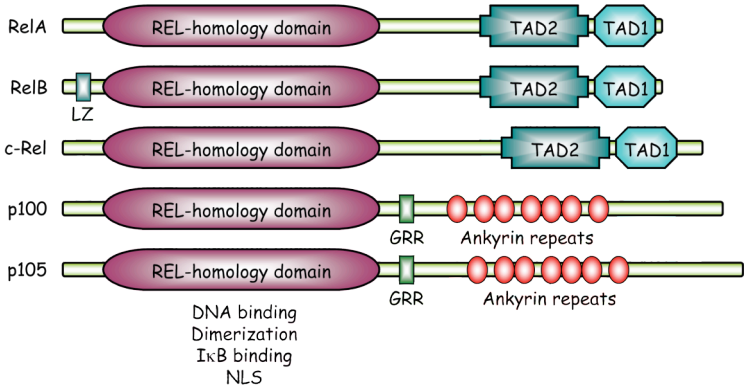

Figure 1.5. The NF-κB family of transcription factors. All mammalian NF-κB members, i.e. RelA, RelB, c-Rel, p100 and p105, contain the N-terminal REL-homology domain (RHD) responsible for DNA binding, dimerization with other members of NF-κB family, IκB binding and nuclear translocation (NLS, nuclear localization sequence). On their C-terminal region RelA, RelB and c-Rel contain transactivation domain (TAD), which makes these members transcriptionally active. The leucine zipper (LZ) of RelB is required for its transactivation activity. p100 and p105 contain IκB-like ankyrin repeat responsible for their cytoplasmic sequestration. Glycine-rich region (GRR) is important for their processing to p52 and p50, respectively. Adopted and modified from (86).

relies on the presence of multiple ankyrin repeats within the C-terminus domain of IκBs that bind and sequester NF-κB in the cytoplasm (89). In the case of transcriptionally active members, IκBs bind to the specific sequence within NF-κB RHD, masking their nuclear localization sequence (NLS). p105 and p100, on the other hand, do not contain TAD, but instead contain ankyrin repeats within their C-terminal region, functioning as IκB molecules. Dissociation of NF-κB from its inhibitors is achieved by proteasome-mediated IκB proteolytic-degradation resulting in nuclear translocation of NF-κB dimers aided by NLS within their RHD.

NF-κB is evolutionary conserved, being extensively studied in different model organisms, including *Drosophila* (90). The role of NF-κB signaling pathway in *Drosophila* was first demonstrated in the context of embryonic development (91, 92). However, soon after the discovery that Toll receptor can activate *Drosophila* immune response, several genes involved in *Drosophila* embryonic development have been also shown to regulate its antimicrobial responses (93). Three *Drosophila* Rel proteins have been described to date, namely Dorsal, Dorsal-related immunity factor (Dif) and Relish. Dorsal and Dif share the C-terminal TAD and are sequestered in the cytoplasm by an IκB-like protein Cactus (94, 95). The third NF-κB member Relish, alike p100 and p105, contains C-terminal ankyrin repeats, which proteolytic degradation precedes its nuclear translocation and transcription of antibacterial peptides (96).

The evolutionary origin and functional conservation of NF-κB are traced back as far as 550 million years to the most ancient arthropod horseshoe crab, *Carcinoscorpius rotundicauda* (Cr) (97). Host defense systems against infection in horseshoe crab consists of granular hemocytes that, when activated by MAMPs, such as bacterial lipopolysaccharide (LPS), secrete various molecules providing host protection against those pathogens (98). In keeping with this findings

TLRs (99) as well as molecules of the NF-κB signaling transduction pathway, i.e. CrNF-κB, CrRelish and IκB-like member, i.e. CrIκB, have also been identified in horseshoe crabs (97). Using bioinformatics approach, NF-κB-like molecules have been identified in the genome of the most recent ancestor to living bilaterians, i.e. cnidarian *Nematostella vectensis* (Nv) (100), or in even older demosponge *Amphimedon queenslandica* (Aq) (101). Moreover, the physiological role of NvNF-κB has been recently demonstrated (102), further arguing for the central importance of NF-κB as a stress-mediating TF regulating homeostasis throughout evolution.

1. Two pathways to NF-κB activation

The first evidences that bacterial LPS can induce the release of NF-κB from its cytoplasmic inhibitor IκB (103) started an era of extensive research on detailed biochemical pathway regulating NF-κB transcriptional activity and its physiological relevance. Ever since, stimulus-induced IκB phosphorylation and degradation has been widely studied and is today perceived as a central mechanism regulating NF-κB activity (see Section II.2.2.). The function of the IκB kinase, regulating the phosphorylation and hence the degradation of IκB molecules, was first assigned to a 700 kDa multi-subunit kinase complex, namely IκB kinase (IKK) signalosome (104, 105). IKK consists of two catalytically active subunits, i.e. IKKα (IKK1) and IKKβ (IKK2) and a regulatory subunit IKKγ (NF-κB essential modulator, NEMO). Depending on the stimuli leading to the IKK activation, the IKK subunits phosphorylating IκB and the NF-κB members associated with IκB, two distinct NF-κB signal transduction pathways have been revealed (see Figure 1.6.).

Stress stimuli generally associated with inflammation, e.g. LPS, tumor necrosis factor (TNF), elicit the so called canonical signal transduction pathway, converging at the level of the IKK complex composed of both

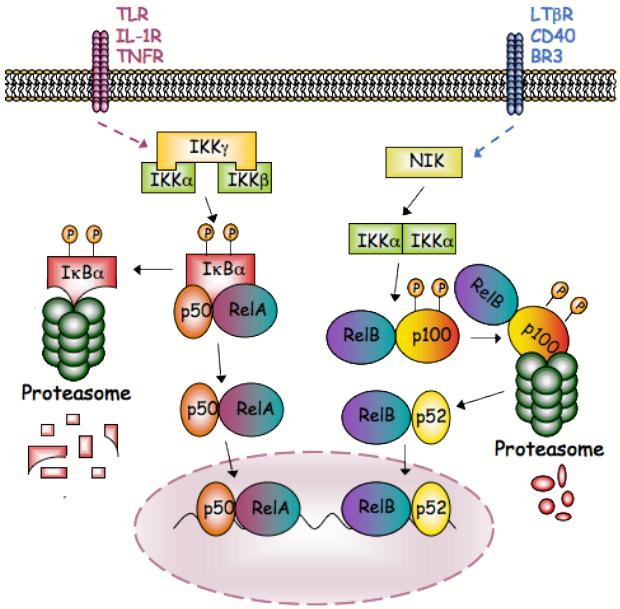

Figure 1.6. Mammalian NF-κB signaling pathway. NF-κBs are ubiquitously expressed as pre-existing transcription factors (TF) sequestered in the cytoplasm via the interaction with their inhibitors IκBs. Stimulus induced IκB degradation leads to NF-κB nuclear translocation and induction of gene transcription. The canonical pathway is generally induced under the stress conditions associated with inflammation (LPS, IL-1, TNF) leading to the activation of the IKK complex composed of both catalytic (IKKα and IKKβ) and a regulatory (IKKγ) subunits, which mediate phosphorylation-dependent IκBα degradation and nuclear translocation of NF-κB dimer, RelA-p50 being prototypical one. On the other hand, the non-canonical pathway is induced by stimuli such as lymhotoxin-β, CD40 and BAFF mainly during the development of secondary lymphoid organs. It is mediated via the NIK-induced activation of IKKα homodimeric complex, leading to the processing of p100 and RelB-p52 nuclear translocation.

catalytic and a regulatory subunit. IKKγ-dependent IKKβ activation is necessary and sufficient for IκBα and/or IκBβ phosphorylation and subsequent degradation, leading to NF-κB dimer (e.g. RelA-p50) nuclear translocation. The physiological significance of canonical pathway has been demonstrated using a loss of function approach in which the genes encoding for IKKβ , IKKγ and RelA were specifically deleted in mice, i.e. *Ikbkb*$^{-/-}$, *Ikbkg*$^{-/-}$ and *Rela*$^{-/-}$ respectively, all of which exhibit embryonic lethality caused by hepatocyte PCD in the developing liver (106-108). Disrupting signaling by TNF in RelA deficient mice (*Rela*$^{-/-}$*Tnfr*$^{-/-}$) restores embryonic development, but results in increased susceptibility to microbial infection, confirming the expected role of NF-κB in proinflammatory gene induction (109). Moreover, IKKβ deficient cells (*Ikbkb*$^{-/-}$) stimulated with proinflammatory cytokines (e.g. TNF, IL-1β) do not induce NF-κB-dependent transcription (107). Together with other similar studies, these findings reveled an irreplaceable role of NF-κB as a cytoprotective TF suppressing PCD, thus uncoupling the proinflammatory effect of TNF from cytotoxicity. The possibility to specifically target NF-κB activation in different cell lineages is, however, starting to reveal a rather tissue-specific and sometimes opposing role of NF-κB in a diversity of inflammatory responses. For example, IKKβ (*Ikbkb*$^{-/-}$) and IKKγ (*Ikbkg*$^{-/-}$) deletion and inhibition of the canonical pathway in epithelial cells results in PCD, disruption of epithelial barrier integrity and increased inflammation caused by commensal bacteria via the activation of tissue Mø arguing for an anti-inflammatory and cytoprotective role for the canonical NF-κB pathway (110, 111). In contrast, specific deletion of IKKβ (*Ikbkb*$^{-/-}$) in neutrophils attenuates PCD, suggesting the pro-apoptotic role of NF-κB in these cells (112, 113). In a model of pneumonia induced by *Streptococcus agalactiae*, on the other hand, IKKβ deletion (*Ikbkb*$^{-/-}$) in lung epithelial cells results in

decreased neutrophil recruitment and bacterial clearance, while enhancing these responses in mice with IKKβ deletion in Mø (113).

In contrast to canonical pathway, other stimuli such as lymphotoxin-β, CD40 and B cell-activating factor (BAFF) activate NF-κB-inducing kinase (NIK), a common denominator of the non-canonical NF-κB signal transduction pathway, which acts irrespectively of IKKβ and IKKγ and is mediated via the IKKα homodimeric complex-dependent processing of p100 and RelB-p52 nuclear translocation (see Figure 1.6.) (114). The non-canonical pathway is involved mainly in the development of secondary lymphoid organs and B-lymphocyte function, a notion supported by a loss of function approach in mice lacking p100 (*Nfkb2*$^{-/-}$), RelB (*Relb*$^{-/-}$) and NIK (*Map3k14*$^{-/-}$) expression (115, 116). It should be noted, however, that studies with *Map3k14*$^{-/-}$ mice, reveled an anti-inflammatory role for RelB as well. *Map3k14*$^{-/-}$ mice develop multiorgan inflammation, a pathology thought to involve impaired T cell function (117).

Though originally thought to act independently from each other, the two pathways can interact at different levels. Genetic studies using mice with conditional deletion of IKKα (*Chuk*$^{-/-}$) and IKKβ (*Ikbkb*$^{-/-}$) uncovered the redundant function of these kinases in mediating canonical NF-κB signaling pathway. This is supported by the notion that only the combined deletion of both kinases (*Chuk*$^{-/-}$*Ikbkb*$^{-/-}$) sensitizes liver parenchymal cells to LPS-induced PCD (118). Moreover, IKKα can phosphorylate canonical IκBs in the absence of IKKβ (118). In the absence of sufficient IKKα activation, on the other hand, excess of p100 can bind to RelA-containing dimers acting as an inhibitor of the canonical pathway (119). As p100 is a RelA-inducible gene, this serves as another negative feedback mechanism involved in the termination of NF-κB-dependent transcription. Moreover, RelB transcription has also been

shown to be RelA-dependent (120), suggesting that the canonical signal transduction pathway can control the non-canonical one.

As in mammals, two similar cascades are present in *Drosophila*, suggesting the functional conservation of the NF-κB signal transduction pathways throughout evolution and also providing *Drosophila* as a model organism to study NF-κB signaling system (see Figure 1.7.). Indeed, our understanding of NF-κB signaling cascades comes in part from studies in *Drosophila*, e.g. Toll receptor that was first identified in *Drosophila* (121-123) and its homologue was identified only thereafter in mammals (124, 125). While the canonical IκB kinase still remains to be identified, activation of the canonical pathway by Toll activation and myeloid differentiation primary response gene 88 (MyD88)-Tube-Pelle complex formation (homologues to mammalian MyD88, an adapter protein and IRAK, respectively) precedes Cactus (homologue to mammalian IκB) phosphorylation and degradation (126-128). This liberates Dorsal and Dif to undergo nuclear translocation leading to the up-regulation of antimicrobial genes such as *Drosomycin* (129). Contrary to mammalian cells, however, Toll ligand in *Drosophila*, Spätzle, is activated in response to fungi and gram-positive bacteria (82, 130). Gram-negative bacterial LPS, on the other hand, triggers the non-canonical Immune deficiency (Imd) signaling cascade, leading to the activation of *Drosophila* IκB kinase complex (IKK) and resulting in Relish activation (see Figure 1.7.) (83, 96, 131, 132). As for p100 and p105, proteolytic degradation of ankyrin repeats within the C-terminal region of Relish is necessary for its nuclear translocation and induction of antibacterial peptides including Diptericin and Defensin (133, 134). As in mammals, synergistic activations of two pathways in *Drosophila* have been recently revealed. Depletion of components acting on the non-canonical Imd pathway can interfere with the canonical Toll induced expression of

antimicrobial genes (135). Moreover, Relish-Dorsal and Relish-Dif dimers have been identified as the most potent inducers of Defensin and Drosomycin, respectively (136).

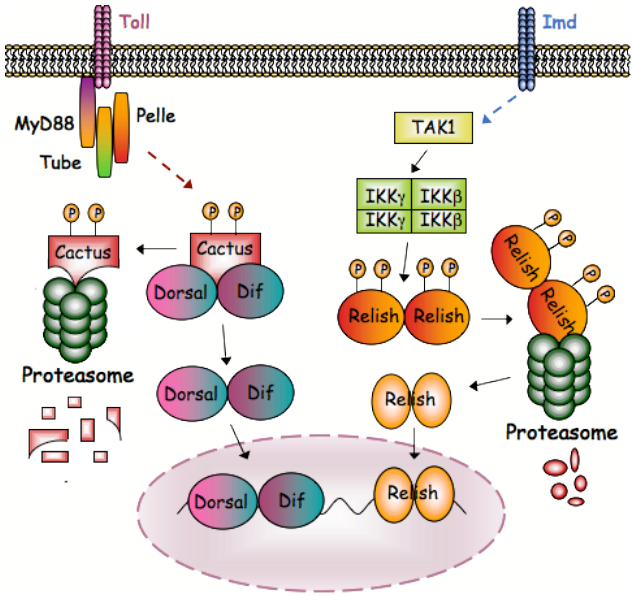

Figure 1.7. *Drosophila* NF-κB signaling pathway. NF-κB family members, signaling pathways leading to their activation and mechanisms regulating their activity are evolutionary conserved. In *Drosophila*, canonical pathway is activated in response to fungi and gram-positive bacteria. Upon Toll activation by Spätzle, MyD88-Tube-Pelle complex formation precedes Cactus phosphorylation and degradation liberating Dorsal and Dif to undergo nuclear translocation leading to the expression of antimacrobial genes. Gram-negative bacterial infection triggers the non-canonical Imd pathway leading to TAK1-induced IKK activation, processing of Relish and its nuclear translocation.

2. Regulation of NF-κB transcriptional activity

The complex role of NF-κB in re-programming gene transcription and hence the expression of numerous inflammatory mediators has been established (137, 138). While the specificity of these responses is known to be largely dependent on different types of stress and targeted tissues, the molecular mechanisms regulating these responses are not fully understood. Thus the transcriptional regulation of the inflammatory response is an important subject of research in many laboratories. Current understanding of mechanisms controlling NF-κB transcriptional activity will be described in this section.

2.1. NF-κB dimerization

Transcriptional response in multi-cellular eukaryotes involves the combinatorial action of multiple TFs and the potential of TFs to oligomerize serves as one mechanism controlling transcriptional activity. Dimeric TFs are grouped into families, which often expand with the organism complexity, providing a mechanism for increased diversity of transcriptional responses (139). As for other evolutionary conserved TFs, NF-κB evolved in a similar manner, from a single TF in demosponge and cnidarians to three members in *Drosophila* and five in mammals, acting as homo- or hetero-dimeric complexes (see Section II). Despite extensive research reveling the specific function of these dimers, their physiological role remains to be fully understood.

Five mammalian NF-κB members can hypothetically form up to fifteen homo- or hetero-dimeric complexes, contributing to the regulation of distinct sets of genes, in that the individual dimers have distinct specificities depending on the stimulus and the cell type responding to that particular stimulus (89). However, some NF-κB members have been

found exclusively in specific dimeric complexes, such as RelB that dimerizes only with p50 and p52 (89). Moreover, NF-κB dimers that do not contain TAD, i.e. p50 and p52 homo- or hetero-dimers, can act as transcriptional repressors (140, 141). Different dimers are preferentially activated by distinct NF-κB signaling cascades. Those activated via the canonical pathway (see Figure 1.6.) include RelA and cRel, which form either homodimers or heterodimers with the transcriptionally inactive p50. While RelA-p50 is the most abundant NF-κB dimer in the majority of cell types, loss of function studies using cRel deficient mice (*Rel*$^{-/-}$) showed an essential role of this NF-κB member for lymphocytes function as well as hematopoetic cell lineages development (142). Furthermore, contrary to RelA-containing dimers, which are constitutively expressed in their non-active form, c-Rel-p50 heterodimers are constitutively active in B cells (143). RelA and cRel, however, can have overlapping functions, in that the combined lack of both RelA and cRel proteins (*Rela*$^{-/-}$*Rel*$^{-/-}$) accelerates the embryonic lethality observed with *Rela*$^{-/-}$ deficient mice (144) (see Section II.1.). The p50 is ubiquitously expressed and present in different NF-κB dimeric complexes. Surprisingly, lack of p50 protein (*Nfkb1*$^{-/-}$) is tolerated during embryonic development, although these mice show multifocal defects in stress responses in that they are highly susceptible to certain microbial challenge, e.g. *Streptococcus pneumoniae*, while they respond normally, e.g. *Haemophilus influenza*, or are even more resistant to others, e.g. encephalomyocarditis virus (145). While RelA and c-Rel containing dimers are the major players of canonical pathway, non-canonical NF-κB signal transduction pathway leads to the activation of the major RelB-p52 heterodimers (see Figure 1.6.), which have an essential role during the development of lymph nodes and B cell maturation.

Upon nuclear translocation, NF-κB dimers bind to the κB sites in

the promoter region of NF-κB-dependent genes, where the conventional binding sequence is represented as KGGRHTYYCC (where K= G or T, H= not G, R= purine, Y= pyrimidine). While most κB sites have a similar affinity for different NF-κB dimers, selective κB sites have been identified for specific dimers, i.e. RGGAGAYTTR site found in the promoter regions of specific genes, i.e. the chemokines *CXCL13* and *CCL19* regulated by RelB-p52 upon activation of non-canonical NF-κB signaling pathway (146). Moreover, c-Rel-RelA heterodimers have been shown to preferentially bind to the consensus sequence, HGGARNYYCC (where N indicates any base), which differs from the κB consensus established for binding RelA-p50 (GGGRNNYYCC) (147).

2.2. NF-κB-IκB interaction

The canonical and best-characterized member of IκB family is IκBα, which under homeostatic conditions binds to the RelA-containing NF-κB dimers such as RelA-p50. As the other members of the IκB family, IκBα contains multiple ankyrin repeats within its C-terminal region, which mask the NLS of RelA, thus restraining its DNA binding and transcriptional activity (see Figure 1.8.). Contrary to RelA, IκBα does not mask the NLS of p50, which together with the nuclear export sequence (NES) of IκBα promotes constant RelA-p50-IκBα shuttling between the cytoplasm and the nucleus (148). Induction of IκBα phosphorylation and its subsequent degradation in response to a variety of stimuli is required for NF-κB nuclear localization, DNA binding and initiation of gene transcription (149). On the other hand, RelA-dependent IκBα re-synthesis is essential for timely termination of NF-κB transcriptional activity (150, 151). While not bearing a traditional NLS, IκBα contains a cis-acting nuclear import sequence within its C-terminal domain that promotes nuclear translocation, association with the promoter bound

RelA and termination of the transcriptional response (152). Given the diversity of physiological responses controlled by NF-κB, it is not surprising that ablation of IκBα and hence increased and sustained NF-κB activity is detrimental. Indeed, mice deficient in IκBα (*Nfkbia*$^{-/-}$) have enhanced expression of proinflammatory mediators and die within 7-10 days of birth with severe inflammatory dermatitis (153).

The canonical IκB family consists of additional proteins, including IκBβ and IκBε (see Figure 1.8.). Unlike IκBα, IκBβ phosphorylation regulates its ability to sequester NF-κB dimers in the cytoplasm (154). While phosphorylated IκBβ binds to NF-κB dimers and masks their NLS thus interfering with nuclear translocation and DNA binding, dephosphorylated IκBβ can bind NF-κB without interfering with DNA binding. As such, binding of dephosphorylated IκBβ can prevent IκBα binding and consequent nuclear export of RelA, thus promoting

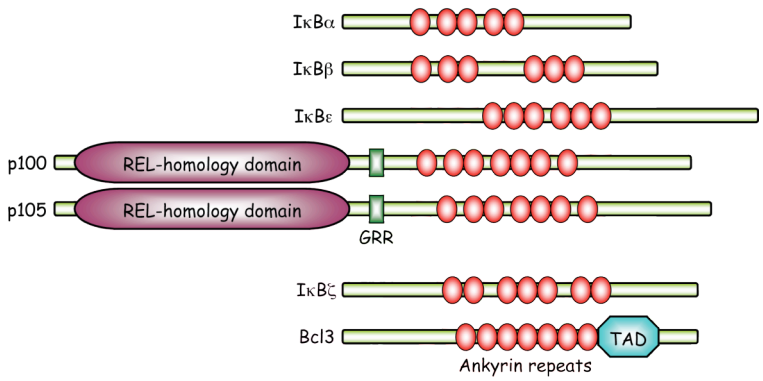

Figure 1.8. The IκB family of NF-κB inhibitory proteins. IκB family of proteins consists of canonical members i.e. IkBα, IkBβ and IkBε, precursor NF-κB members, i.e. p100 and p105, and two atypical members, i.e. IkBζ and Bcl-3. All IκB members contain multiple ankyrin repeats within their C-terminal region, whose degradation is a prerequest for NF-κB transcriptional activity. Bcl-3 contains a TAD, capable of driving NF-κB-dependent transcription. Adopted and modified from (86).

persistent NF-κB transcriptional activity (155). On the other hand, IκBε contains a NES, can bind RelA and remove it from the promoter region of NF-κB-dependent genes, thus providing an additional negative feedback loop regulating NF-κB activity (156). Delayed kinetics of IκBβ and IκBε degradation and RelA-dependent re-synthesis of these IκB isoforms is probably the major functional characteristic determining these IκB members, suggesting their role in the late phase of the NF-κB-dependent transcription (157, 158). This is suggested by the observation that replacing IκBα coding region with that of IκBβ, results in normal kinetics of NF-κB activation and termination (159).

The precursor NF-κB members p100 and p105, termed IκBδ and IκBγ, respectively, function both as NF-κB members as well as their inhibitors (see Figures 1.5. and 1.8.) (89). Cleavage of p100 and p105 leads to the production of nuclear NF-κB member p52 and p50, respectively (160-164). Both p100 and p105 can bind other NF-κB members, inhibiting their transcriptional activity (165, 166). Expression of p100 is associated primarily with RelB, stabilizing and sequestering RelB-containing dimers in the cytoplasm (167). Processing of p100 following stimulation, releases RelB-p52 dimers leading to their nuclear translocation (see Figure 1.6.) (114). RelA-dependent transcription and subsequent expression of p100 can also bind and inhibit the transcriptional activity of RelA-p50 heterodimers, contributing to the termination of NF-κB driven transcription (119). Constitutive processing of p105, on the other hand, results in the production of p50, the main partner of RelA-containing dimers (168). In addition, p105 can bind other NF-κB dimers suggesting its role as a general inhibitor of NF-κB transcriptional activity (165).

The IκB family of proteins contains two additional atypical members, namely IκBζ and Bcl-3, which can bind NF-κB and regulate its

transcriptional activity (see Figure 1.8.). Expression of IκBζ and Bcl-3 is restricted to the nucleus and it remains unclear how these proteins regulate NF-κB transcriptional activity. Both IκBζ and Bcl-3 associate preferentially with p50 or p52 homo- or hetero-dimers enhancing NF-κB activity (169, 170). Bcl-3 contains a TAD, providing a transcriptional activity to otherwise repressors of NF-κB-dependent transcription (171). On the other hand, IκBζ does not contain an apparent TAD, but has been suggested to induce gene transcription, i.e. *Il6*, in response to stimuli such as IL-1 or LPS (169). Alternatively, binding of IκBζ and Bcl-3 to p50 or p52 homo- or hetero-dimers could help replacing these dimers with the transcriptionally active NF-κB members on the promoter regions of NF-κB-dependent genes (169).

2.3. NF-κB post-translational modifications

While NF-κB dimerization and binding to IκBs serve as central components controlling its transcriptional activity (see Sections II.2.1. and II.2.2.), these regulatory mechanisms cannot explain alone the wide range of specificity provided by the NF-κB system (172). One of the mechanisms via which this is achieved is through post-translational modification of different NF-κB family members. These comprise covalent modifications of amino acids, providing a general mechanism increasing protein functional diversity (173). It is often the combinatorial usage and competition between distinct post-translational modifications of an individual protein that offer the potential for specificity. Some of the most common post-translational modifications include phosphorylation, acylation, e.g. acetylation and ubiquitination, alkylation, e.g. methylation, glycosylation and oxidation (174). All of these can influence protein structure and function by various ways, modulating protein interaction

with other partners including proteins, lipids or DNA.

In the context of NF-κB system, post-translational modifications can alter the activity of various core components of NF-κB signaling pathway, e.g. phosphorylation of IκB promotes its ubiquitination and proteolytic degradation. While post-translational modifications can target RelA directly, regulating its transcriptional activity (175, 176), the physiological relevance underlying these modifications remains largely unknown. The biological significance of RelA post-translational modifications is however strongly supported by the fact that these can regulate the transcriptional activity of *Drosophila* Rel protein Dorsal, targeting evolutionary conserved amino acid residues (177). The biological role of RelA post-translational modifications will be addressed in more details below.

2.3.1. RelA phosphorylation

Phosphorylation remains probably the best characterized of all post-translational modifications (178). It consists essentially on the addition of a negatively charged phosphate group usually on a serine (S), threonine (T) or tyrosine (Y) residues via the activity of protein kinases (173). This induces conformational changes of proteins, often exposing the hidden hydrophobic domains for the interaction with other partners, including proteins, lipids or DNA. The same is true for RelA for which several protein kinases have been shown to target specific phospho-acceptors within its RHD as well as TAD, activating or repressing its transcriptional activity (see Figure 1.9.) (175). The first to be identified, i.e. S276, is targeted by protein kinase A (PKA), regulating RelA transcriptional activity (179, 180). Phosphorylation of S276 promotes conformational modifications of RelA inducing its interaction with the transcriptional co-activators such as cyclic AMP-responsive element binding protein

(CREB)-binding protein (CBP)/p300 (181), while diminishing binding of transcriptional co-repressor histone deacetylase HDAC1 (182). The physiological relevance of RelA S276 phosphorylation is illustrated by the finding that mice expressing the constitutively active form of RelA, mimicked by S276D mutation, display systemic increase in proinflammatory cytokines and chemokines induced by TNF signaling succumbing to hyperinflammatory conditions within 8-20 days of birth (183). Generation of mice expressing the hypophosphorylated RelA, mimicked by S276A point mutation, however, revealed a rather complex role of RelA S276 phosphorylation in regulating gene transcription (184). Contrary to the RelA deficient mice (*Rela*$^{-/-}$) that die from massive

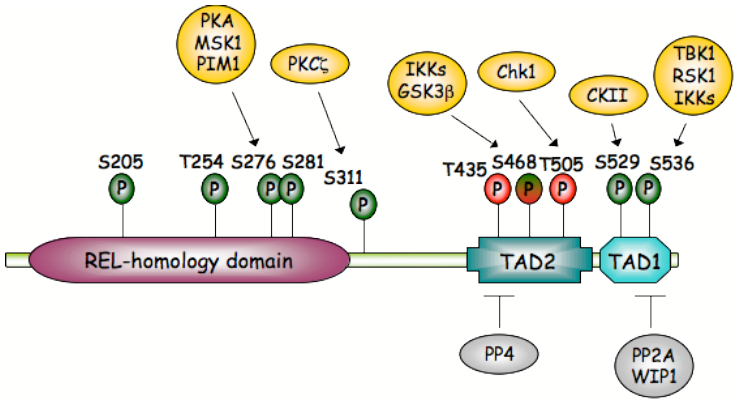

Figure 1.9. RelA phosphorylation. Schematic representation of various known kinases and phosphatases regulating RelA transcriptional activity via modulation of its phosphorylation on different serine and threonine residues. Phosphorylation that promotes RelA transcriptional activity is represented in green, while that inhibiting its activity in red. S468 phosphorylation has been shown to both promote and inhibit RelA transcriptional activity in the context-dependent manner. PKA, protein kinase A; MSK1, mitogen- and stress-activated protein kinase-1; PKCz, protein kinase Cζ; IKK, IκB kinase; GSK3b, glycogen-synthase kinase-3β; Chk1, checkpoint kinase 1; CKII, casein kinase II; TBK1, TANK binding kinase; RSK1, ribosomalsubunit kinase-1; PP protein phosphatase; WIP1, wild-type p53-induced phosphatase.

hepatocyte PCD during the embryonic development (see Section II.1.), mice expressing RelA S276A mutant die from multifaceted developmental abnormalities. This can be explained to some extent by the recruitment of the transcriptional co-repressor HDAC to the promoter region of some NF-κB-dependent genes, repressing the transcription of subset of genes surrounding putative NF-κB sites (184).

RelA interaction with CBP/p300 can occur in response to RelA phosphorylation by several kinases, including PKA, mitogen- and stress-activated kinase-1 (MSK1), IKK and protein kinase C (PKC) ζ, which phosphorylate specific RelA residues, e.g. S276, S311, S536 (see Figure 1.9.) (185-188). The existence of distinct pathways targeting different RelA phospho-acceptors and providing a similar biological outcome, i.e. enhanced gene transcription, argues strongly for the biological relevance of this post-translational modification in the regulation of NF-κB transcriptional activity. In keeping with this notion, regulation of NF-κB activity by phosphorylation also occurs in *Drosophila* in which the PKA homologue, i.e. DmPKA, phosphorylates the RelA homologue Dorsal on conserved S276 site, i.e. Dorsal S312, controlling its nuclear translocation (177, 189).

Other than interaction with transcriptional cofactors, RelA phosphorylation can also promote other RelA post-translational modifications, i.e. acetylation, methylation and ubiquitination, as well as modulate its nuclear translocation, DNA binding and stability (176). For example, RelA S468 phosphorylation by IKKε promotes RelA translocation to specific subnuclear compartments (see Section II.2.5.), decreasing its transcriptional activity (190). RelA S468 phosphorylation can also promote RelA ubiquitination by the copper metabolism MURR1 (COMM) domain-containing protein (COMMD1) leading to RelA proteasomal degradation and decrease of NF-κB transcriptional activity

(191). In addition, RelA S536 phosphorylation by ribosomal protein S6 kinase polypeptide 1 (RSK1) or TANK-binding kinase 1 (TBK1) diminishes RelA affinity for IκBα, thus increasing its DNA binding (192).

While the phosphorylation status of RelA results from opposing actions of kinases and phosphatases, little is known about how dephosphorylation regulates RelA transcriptional activity. While two phosphatases, i.e. protein phosphatase 2A (PP2A) and wild-type p53-induced phosphatase 1 (WIP1), can dephosphorylate RelA S536, decreasing its transcriptional activity (193), RelA S435 dephosphorylation by phosphatase 4 increases its transcriptional activity (194). Identification of novel phosphatases as well as specific mechanisms underlying their activity will be therefore needed to fully understand the effect of reversible RelA phosphorylation on the expression of distinct gene profiles.

2.3.2. RelA acetylation

Acetylation, initially discovered on histones, consists on the addition of an acetyl group to the amino-terminal residues or to the ε-amino group of lysine (K) residues on proteins (173). By neutralizing the positively charged protein region, acetylation regulates multiple functions of proteins including DNA binding, interaction with other proteins, subcellular localization and stability (195). The acetylation status of proteins results from the opposing action of histone acetyltransferases (HATs) and HDAC, enzymes that catalyze the addition and removal of an acetyl group, respectively. Alike several other TFs, RelA acetylation controls its transcriptional activity. It took couple of years to identify the inducible acetylation of RelA by CBP since their interaction was first established (196, 197). CBP/p300 is to date the best characterized RelA acetyltransferase in that it can acetylate seven different RelA K residues

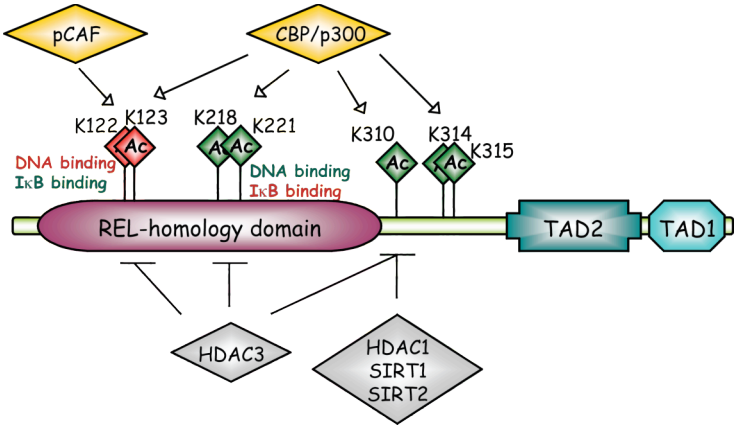

Figure 1.10. RelA acetylation. Schematic representation of known acetyl transferases and deacetylases regulating RelA transcriptional activity via modulation of its acetylation on different lysine (K) residues. Acetylation that promotes RelA transcriptional activity is represented in green, while that inhibiting its activity in red. pCAF, p300/CBP-associated factor; CBP, CREB-binding protein; HDAC, histone deacetilase; SIRT, sirtuin.

(see Figure 1.10.) (176). Some of these are also acetylated by the p300/CBP-associated factor (P/CAF) (198). Acetylation is mostly a nuclear event (176), controlling RelA transcriptional activity as well as the duration of NF-κB activation via regulation of DNA binding and association with IκBα. While K310, K314 and K315 acetylation enhances RelA transcriptional activity without affecting DNA binding (199, 200), K218 and K221 acetylation increases NF-κB transcriptional activity, inhibiting its removal from DNA by newly synthesized IκBα (199). In contrast, K122 and K123 acetylation reduce RelA transcriptional activity by decreasing DNA binding in an IκBα-independent manner (198). HDAC3 is the best-characterized RelA deacetylase, regulating its transcriptional activity by targeting RelA K122, K123, K221 and K310

(197, 198). Three other deacetylases, i.e. HDAC1, sirtuin 1 (SIRT1) and SIRT2, have been shown to deacetylate K310, decreasing RelA transcriptional activity (201, 202). Overall, these findings suggest that acetylation is important in regulating duration of RelA transcriptional activity and future studies will be needed to reveal physiological implications of RelA acetylation in maintaining homeostasis.

2.3.3. RelA methylation

The ε-amino group of protein K residues can also undergo, in addition from acetylation, mono-, di- and tri-methylation, a process that consists on the addition of methyl groups increasing protein hydrophobicity and disrupting intra- or intermolecular hydrogen-bond interactions or creating new protein binding sites (173). RelA can undergo methylation by different histone lysine methyltransferases (HKMTs) that influence RelA interaction with transcriptional cofactors (see Figure 1.11.). For example, under the homeostasis, RelA K310 is monomethylated by suppressor of variegation-enhancer of zeste-trithorax (SET) domain containing protein 6 (SETD6). This promotes RelA interaction with the transcriptional co-repressor histone methylase G9A-like protein (GLP), which can methylate histone H3 Lys9, repressing gene transcription on a subset of NF-κB-dependent genes (203). In addition, RelA methylation can promote other post-translational modifications, as illustrated for RelA K314 and K315 mono-methylation mediated by SET domain HKMT 9 (Set9) that promotes RelA ubiquitination and proteasomal degradation thus decreasing NF-κB dependent transcription (204). RelA methylation can also promote NF-κB transcriptional activity, e.g. K218 and K221 methylation by nuclear receptor-binding SET domain-containing protein 1 (NSD1) (205). While RelA methylation status results from the opposing effect of HKMT and histone lysine demethylases (HKDM), only one

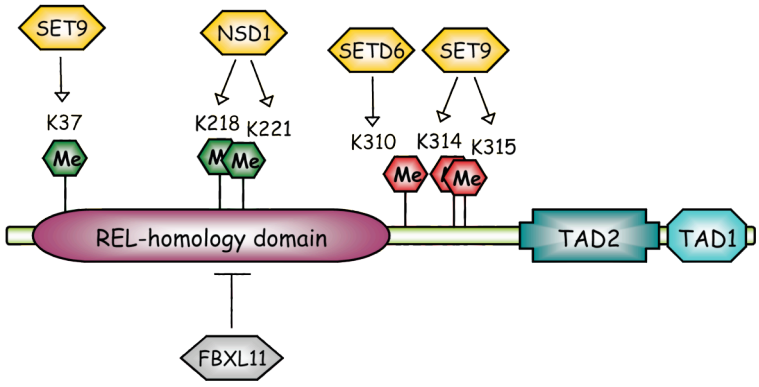

Figure 1.10. RelA methylation. Schematic representation of various known methyltransferases and demethylases regulating RelA transcriptional activity via modulation of its methylation on different lysine residues. Methylation that promotes RelA transcriptional activity is represented in green, while that inhibiting its activity in red. SETD, suppressor of variegation-enhancer of zeste-trithorax domain containing protein; NSD1, nuclear receptor-binding SET domain-containing protein 1; FBXL11, F-box and leucine-rich repeat protein 11.

HKDM has been identified to date, i.e. F-box and leucine-rich repeat protein 11 (FBXL11) that demethylates RelA K218 and K221, decreasing cell proliferation and colony formation of HT29 cancer cells (205). Further experiments will be needed to identify specific demethylases for different K residues providing new insights into the regulation of NF-κB by reversible methylation.

2.3.4. RelA ubiquitination

Ubiquitination consists on the addition of ubiquitin to a side chain of a protein K residue via a tree step process: i) ubiquitin activation by an ubiquitin-activating enzyme (known as E1), ii) transfer of activated ubiquitin to the ubiquitin conjugating enzyme (known as E2) and iii)

transfer of ubiquitin from E2 to a K residue in the target protein, mediated by an ubiquitin-protein ligase (known as E3) (206). Mono- or poly-ubiquitination regulates vast array of cellular functions some of which via modulation of NF-κB activity (207). IκB ubiquitination and subsequent proteasomal degradation has been long known to control NF-κB nuclear translocation and activation (see Section II.2.2.). Ubiquitination can also directly target RelA (see Figure 1.12.), first illustrated for the ubiquitin ligase suppressor of cytokine signaling 1 (SOCS1), leading to its degradation and contributing to termination of NF-κB transcriptional activity (208, 209). While initially associated with protein degradation via the 26S proteasome, RelA ubiquitination can induce its lysosomal degradation as well (210). Moreover, RelA ubiquitination and degradation are often associated with its trafficking to specific insoluble nuclear compartments such as shown for RelA translocation into promyelocytic leukemia (PML) nuclear bodies

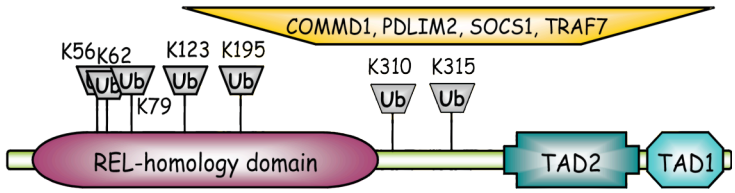

Figure 1.12. RelA ubiquitination. Schematic representation of the identified lysine residues undergoing ubiquitination as well as four known ubiquitin ligases that regulating RelA transcriptional activity via modulation of its ubiquitination. COMMD, copper metabolism MURR1 domain-containing protein; PDLIM, protein containing postsynaptic density 65-disc large-zonula occludens 1 (PDZ) and abnormal cell lineage 11-islet 1-mechanosensory abnormal 3 (LIM) domains; SOCS, suppressor of cytokine signaling; TRAF, TNF receptor associated factor.

mediated by the ubiquitin ligase PDLIM2, a nuclear protein containing postsynaptic density 65-disc large-zonula occludens 1 (PDZ) and abnormal cell lineage 11-islet 1-mechanosensory abnormal 3 (LIM) domains (see Section II.2.5.) (211). Ubiquitination of Rel proteins seems to be an evolutionary conserved mechanism regulating NF-κB activity. In keeping with this notion, loss of function of SkpA-containing ubiquitin ligase complex in *Drosophila* results in the increased expression of *Drosophila* NF-κB member Relish and hence higher expression of its target antimicrobial gene *Diptericin* (134). Non-degradative forms of RelA ubiquitination have also been identified, suggesting a regulatory role for ubiquitination independently of proteolysis (212).

2.4. NF-κB-DNA binding

Eukaryotic genes are tightly embedded around histones forming complex and dynamic structures known as chromatin, which in its default state may limit TF accessibility to promoters, thus acting as a transcriptional barrier regulating gene expression (213, 214). Changes in chromatin structure are therefore often required to increase promoter access for TF as well as components of the basic transcriptional machinery allowing the initiation of transcription. These include chromatin remodeling as well as reversible histone tail post-translational modifications, such as provided upon phosphorylation, acetylation, methylation and ubiquitination (215). With regard to promoter recruitment, there seem to be two kinds of NF-κB-dependent genes: those that are readily accessible and those that require chromatin remodeling (216). NF-κB phosphorylation induces conformational changes allowing its interaction with a transcriptional activator HAT CBP/p300, which can acetylate histone tails, a critical step required for the expression of a subset of NF-κB target genes (181, 217-219).

Besides CBP/p300, NF-κB can recruit other HATs as well as HDACs, such as P/CAF and HDAC1, respectively, regulating the accessibility of chromatin for transcriptional machinery (182, 220). As discussed above, opposing action of HATs and HDACs also regulate the strength and duration of the NF-κB transcriptional response, influencing its DNA binding as well as its interaction with IκBα (see Section II.2.3.2.). Besides acetylation, histones can undergo other post-translational modifications such as phosphorylation, influencing the promoter accessibility for TF and components of the transcriptional machinery in a similar way as histone acetylation (221). Activation of IKKα in response to TNF leads to its recruitment to the promoter of some NF-κB dependent genes in a complex with CBP and RelA, leading to histone phosphorylation (222, 223). Two other kinases, i.e. PKA and MSK1, which also regulate NF-κB transcriptional activity (see Section II.2.3.1.), can mediate histone phosphorylation in response to different stimuli (224, 225). Thus the combined effect of NF-κB and histones post-translational modifications is probably needed for a stimulus specific pattern of gene expression (226).

2.5. NF-κB subnuclear compartmentalization

Cell nucleus comprises a heterogeneous group of complex and highly dynamic subnuclear compartments collectively known as nuclear bodies (227). Other than chromosomes and nucleoli, these include Cajal bodies, splicing factor compartments (i.e. speckles), PML bodies and many others that are often associated with specific gene loci regulating replication and transcription. As such, the nucleoli are constitutively associated with genes encoding 35S ribosomal (r)RNA and are involved in rRNA transcription, processing and ribosomal subunit assembly (228). On the other hand, Cajal bodies, speckles and PML bodies are only

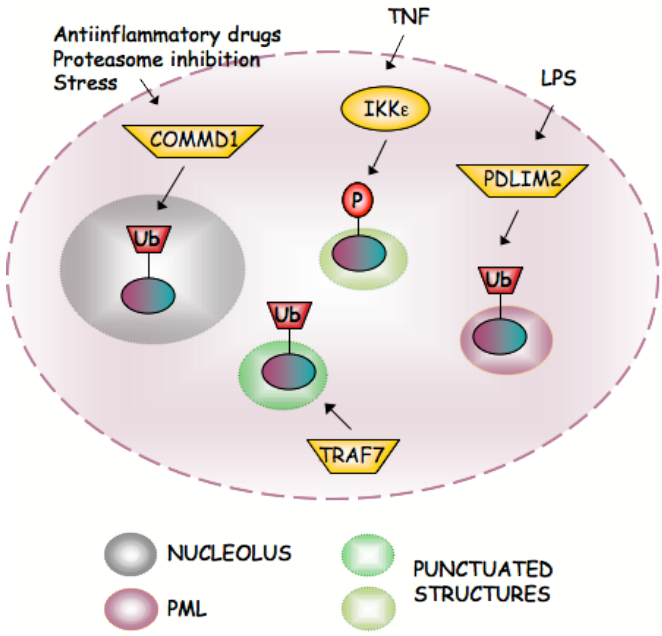

Figure 1.12. RelA subnuclear compartmentalization. In response to stress, anti-inflammatory drugs and proteasome inhibition, COMMD1-mediated RelA ubiquitination precedes its nucleolar translocation. Upon LPS stimulation, RelA undergoes PDLIM2-mediated ubiquitination and translocation to promyelocytic leukemia protein (PML) nuclear bodies. TRAF7 mediated RelA ubiquitination and IKKε-mediated RelA phosphorylation lead to RelA translocation to nuclear punctuated structures.

transiently associated with specific genomic loci and are involved in biogenesis of small nuclear ribonucleoproteins, storage of splicing factors and control of transcription, respectively (229-231). Translocation of TFs, including NF-κB, to different nuclear bodies is now recognized as a mechanism controlling TF access to gene promoters, regulating gene transcription (232). Protein post-translational modifications such as ubiquitination is emerging as an important mechanism regulating TF subcellular localization, including for RelA (see Figure 1.13.), whose

translocation and sequestration in the nucleoli induced by stress, anti-inflammatory drugs and proteasome inhibition can result in down-regulation of NF-κB-dependent transcription and cell apoptosis (233-235). On the other hand, RelA ubiquitination and proteasomal degradation involved in the termination of NF-κB transcriptional activity can occur in PML nuclear bodies (211). Several other proteins involved in NF-κB-dependent transcription (e.g. CBP/p300, IKKε) can also be sequestered in PML nuclear bodies (190, 236), suggesting that nuclear bodies may enable the fine control of NF-κB transcriptional activity in the nucleus presumably independently of RelA degradation.

3. Identification of NF-κB regulatory proteins by RNA interference screening

RNA interference (RNAi) has emerged as an efficient method to suppress gene expression specifically, thus allowing the assessment of gene function. Although RNAi gene screens came into common use there are still significant technical challenges that devoid this approach to be commonly used in mammalian cells. This is not the case for *Drosophila* cells, which incorporate double stranded RNA (dsRNA) with nearly 100% efficiency, acting as a potent RNAi. Sequencing of *Drosophila* genome (237) and generation of dsRNA libraries targeting all *Drosophila* genes individually (238) allows functional screen of large sets of genes in *Drosophila* cells. Various *Drosophila* and human genes as well as signal transduction pathways are evolutionary conserved and around 60% of genes involved in human diseases have a homologue in *Drosophila* (239). Major components of the NF-κB signal transduction pathway and mechanisms controlling NF-κB transcriptional activity are also conserved between *Drosophila* and mammalian cells (see Sections

II. and II.1.). Gene screens in *Drosophila* cells have been used successfully to identify several evolutionary conserved processes regulating NF-κB activity, e.g. the involvement of G protein-coupled receptor kinase 2 (Gprk2) (mammalian G protein-coupled receptor kinase 5, GRK5), a Cactus (mammalian IκB homologue) interacting protein (240) as well as Defense repressor 1 (Dnr1) (mammalian inhibitor of apoptosis protein, IAP) (241) in the regulation of *Drosophila* NF-κB activity. Using this approach, we identified CCT as an evolutionary conserved regulator of NF-κB activity (see Chapter II).

3.1. Chaperonin containing TCP-1 (CCT)

Chaperonins are one of the major classes of folding-assisting proteins found in all living organisms being essential for organism viability. There are two structurally distinct groups of chaperonins: group I chaperonins, such as *Escherichia coli* GroEL, which are found in prokaryotic cells and endosymbiotic organelles, and group II chaperonins, such as CCT, found in Archea and eukaryotic cells (242). CCT is a ubiquitously expressed multimeric protein complex involved in protein folding as well as assembly of protein complexes in an ATP-dependent manner. It consists of eight subunits (CCTα-θ) that share domains and amino acid sequences conserved across species (243-245). Though originally described as a cytosolic protein involved in actin and tubulin folding (246, 247), a range of non-cytoskeletal CCT substrates have now been identified (248). These include von Hippel-Lin (VHL)-elongin BC tumor suppressor complex (249), cell-division cycle protein 20 (Cdc20) (250), sphingosine kinase 1 (251), Polo-like kinase 1 (PLK-1) (252), HDAC3 (253), huntingtin (254), cyclin E (255). This supports the notion that CCT is involved in various cellular functions such as cell cycle, transcription,

chromatin remodeling and protein degradation. Recessive mutation in CCTδ subunit results in sensory neuropathy (256), while point mutations in the CCT-binding site of the VHL, leading to its severe protein misfolding are found in the renal cell cancer (249). While not directly implicated in controlling NF-κB activity, CCT is required for HDAC3 activity that plays a critical role in NF-κB-dependent transcription (253). Moreover, other CCT substrates, such as VHL and PLK-1, have also been shown to regulate NF-κB transcriptional activity (257, 258). This supports the hypothesis that CCT might regulate NF-κB activity, which we found to be the case (see Chapter II).

III. Objectives

The overall goal of this work was to provide a novel insight into the regulatory mechanisms controlling NF-κB transcriptional activity. More specifically, we have focused on:

1. Identifying evolutionary conserved genes regulating RelA transcriptional activity using an RNAi based gene screen in *Drosophila* cells.

2. Characterizing the crosstalk between different mechanisms regulating RelA transcriptional activity, i.e. RelA phosphorylaton and its subnuclear localization.

3. Determining whether stress responsive genes that play a central role in the control of inflammatory responses regulate NF-κB transcriptional activity.

CHAPTER II
REGULATION OF NF-κB ACTIVITY BY ACETYLATION *

*Adapted from **Pejanovic N**, Hochrainer K, Liu T, Aerne BL, Soares MP, and Anrather J. *Regulation of NF-κB transcriptional activity via p65 acetylation by the chaperonin containing TCP1 subunit η.*

Manuscript in preparation

I. Summary

The nuclear factor kappa B (NF-κB) family of transcription factors (TF) is central to inflammation and immunity. Post-translational modifications of the NF-κB p65/RelA family member regulate its transcriptional activity. The purpose of this study was to identify and characterize evolutionary conserved genes modulating p65/RelA activity. We identified chaperonin containing TCP1 subunit η (CCTη) as a regulator of the transcriptional activity of *Drosophila* Rel proteins, Dorsal and Dorsal-related immunity factor (Dif), using a RNA interference screening approach. CCTη was also found to regulate NF-κB-driven transcription in mammalian cells, acting in a promoter-specific context, downstream of IκB kinase (IKK). CCTη knockdown repressed *IκBα* and *CXCL2/MIP2* transcription during the early phase of NF-κB activation while impairing the termination of *CCL5/RANTES* and *CXCL10/IP10* transcription, the later effect being associated with increased DNA binding and reduced p65/RelA acetylation. We identified lysine (K) 122 and K123 as the target residues mediating the CCTη-driven p65/RelA regulation. Finally, CCTη was found to enhance cyclic AMP-responsive element binding protein (CREB)-binding protein (CBP) activity, probably facilitating NF-κB removal from DNA and hence terminating NF-κB activity. We propose that CCTη regulates NF-κB activity in a manner that resolves inflammation.

II. Background

The NF-κB family of eukaryotic TF controls the expression of a large number of genes regulating inflammation and immunity as well as developmental processes including cellular growth and apoptosis (259).

Unfettered NF-κB activation has been associated with the pathogenesis of a number of inflammatory diseases (84). In their active form, NF-κB proteins are nuclear homo- or hetero-dimeric complexes composed of p65/RelA, RelB, cRel, p105/p50, and p100/p52. The prototypical and most ubiquitously expressed NF-κB dimer is composed of p50 and p65 subunits where p65 is the main transcriptional activator (260).

Under homeostatic conditions, p65 activity is constitutively repressed by its interaction with the cytoplasmic NF-κB inhibitors (IκB) (261). While inducible degradation of IκB molecules is a central mechanism regulating p65 transcriptional activity, post-translational modifications also regulate its activity (175, 176). These include phosphorylation, which modulates DNA binding, interactions with other proteins as well as p65 stability (175). Phosphorylation often precedes other post-translational modifications such as acetylation and ubiquitination (181, 187, 191, 208). For example, p65 phosphorylation at serine (S) 276 facilitates interaction with CBP/p300 and diminishes histone deacetylase 1 (HDAC1) binding leading to p65 acetylation (182). Acetylation is mostly a nuclear event (176), controlling p65 transcriptional activity (197, 199, 200, 262) as well as the duration of NF-κB activation via regulation of DNA binding (198, 199) and association with IκBα (199). Specific p65 K residues may be preferentially targeted by different histone acetyltransferases (HAT) that include CBP, p300 and p300/CBP-associated factor (P/CAF) (198-200). The acetylation status of p65 is controlled by the opposing activities of HATs and HDACs including HDAC1 (201), HDAC3 (197, 198), sirtuin 1 (SIRT1) (263), and SIRT2 (202). Beside acetylation, p65 K residues can be modified by methylation and ubiquitination resulting in altered transcriptional activity or proteasomal degradation (204, 209, 211, 264, 265).

Rel proteins and the signal transduction pathways leading to their activation are highly evolutionary conserved. As in mammals, *Drosophila* p65 homologues Dorsal and Dif are constitutively inhibited by the IκB like molecule Cactus (94), (266). Toll receptor activation leads to Cactus degradation, Dorsal and Dif nuclear translocation and transcription of NF-κB dependent genes such as *Drosomycin* and *Cecropin* (82, 267, 268). Little is known about the impact of post-translational modifications on Dorsal and Dif transcriptional activity. Yet, several phospho-acceptors as well as K residues targeted for post-translational modifications in p65 are conserved in Dorsal and Dif. Supporting this view, S312 and S317 phosphorylation can control Dorsal nuclear translocation (177, 189).

We set to identify evolutionary conserved genes regulating NF-κB-dependent gene transcription using a functional RNA interference (RNAi) based gene screen in *Drosophila* cells. We reveal the CCTη as a novel gene regulating NF-κB acetylation and activity.

III. Results

1. Inhibition of Tcp-1/CCTη expression modulates NF-κB-dependent reporter activity

To identify evolutionary conserved genes regulating NF-κB pathway, we employed a dsRNA library targeting 265 putative *Drosophila* kinases and kinase regulatory proteins. *Drosomycin*-driven *luciferase* (*Drs-luc*) expression can be induced by a constitutively active form of Toll (Toll 10b) as well as by *Drosophila* p65 homologues Dorsal and Dif (135, 269). *Drs-luc* expression increased significantly in *Drosophila* S2 cells co-transfected with Toll 10b, Dorsal or Dif, as compared to cells transfected with *Drs-luc* alone (see Figure 2.1.a). We used the same assay to perform a RNA interference (RNAi) based gene screen in S2

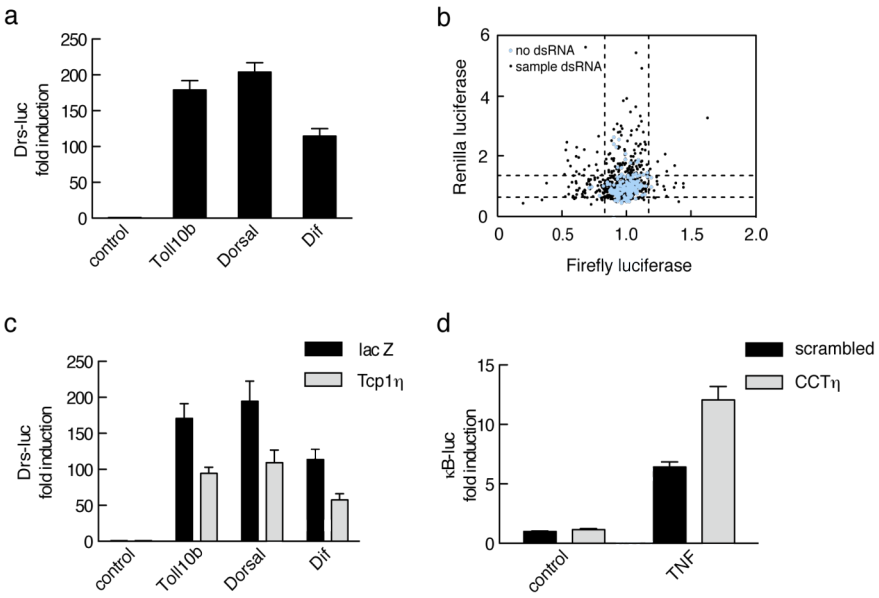

Figure 2.1. Identification of Tcp-1/CCTη as a novel gene regulating NF-κB transcriptional activity. (a) S2 cells were transiently co-transfected with the *Drs-luc* reporter plus or minus Dorsal, Dif or Toll 10b and exposed to LacZ dsRNA. Illustrated data represent mean firefly luciferase normalized to Renilla luciferase units ± SEM (n≥11 derived from four independent experiments) **(b)** S2 cells were transiently co-transfected with the *Drs-luc* reporter plus Toll 10b and seeded into wells of a 384-well plate containing 265 *Drosophila* kinases and kinase regulatory subunits. Firefly and Renilla luciferase values from two independent experimental plates were plotted. Control wells not containing dsRNA are illustrated with light gray diamonds. **(c)** S2 cells were transiently co-transfected as in (a) and exposed to Tcp-1η or LacZ dsRNA. Illustrated data represent mean firefly luciferase normalized to Renilla luciferase units ± SEM (n=9, derived from three independent experiments). **(d)** HEK293 cells were transiently transfected with κ*B-luc* reporter together with CCTη or scrambled shRNA, incubated for three days and stimulated with TNF (10 ng/ml, 6 h) or left untreated (control). Results shown are mean firefly luciferasenormalized to β-galactosidase units ± SEM (n≥7, derived from three independent experiments).

cells transiently transfected with *Drs-luc* reporter, Toll 10b expression construct and a constitutively active *pAct5-Renilla* reporter. Firefly and Renilla luciferase values were plotted and dsRNA treatments that significantly modulated firefly luciferase expression (values lying outside the mean ± 2SD) with no effect on Renilla luciferase values were further validated (see Figure 2.1.b). We identified chaperonin containing Tcp-1 subunit eta (Tcp-1η), an evolutionary conserved gene not previously implicated in regulation of NF-κB signaling. We confirmed that Tcp-1η regulates NF-κB activity in a reporter assay in S2 cells. Tcp-1η dsRNA inhibited Toll 10b-, Dorsal- and Dif-induced *Drs-luc* expression, as compared to control LacZ dsRNA (see Figure 2.1.c).

The mammalian Tcp-1η homologue, CCTη (CCT7), is a member of the type II chaperonins. To investigate whether CCTη controls mammalian NF-κB signal transduction pathway we assessed its effect on TNF-induced NF-κB reporter activity. HEK293 cells were transiently co-transfected with a *κB-luc* reporter and CCTη shRNA or scrambled shRNA as a control. In contrast to S2 cells, CCTη knockdown in mammalian cells enhanced TNF-driven reporter activity, as compared to scrambled shRNA transfected controls (see Figure 2.1.d). The same result was observed when two other CCT subunits, CCTα (TCP1) and CCTζ (CCT6A), were targeted by shRNA (data not shown), suggesting the involvement of the intact chaperonin CCT complex in the regulation of NF-κB activity in mammalian cells.

2. CCTη modulates NF-κB-dependent gene expression

The canonical NF-κB signal transduction pathway can be activated via different receptors, including tumor necrosis factor receptor (TNFR) and the Interleukin-1 receptor (IL-1R) that elicit signal transduction pathways converging at the level of the IKK complex (270). To assess whether

CCTη regulates both the TNFR and IL-1R pathways we analyzed mRNA expression of NF-κB target genes by quantitative RT-PCR in HeLa cells transfected with CCTη or scrambled siRNA. We found that CCTη affects NF-κB-dependent gene expression in a promoter-specific context.

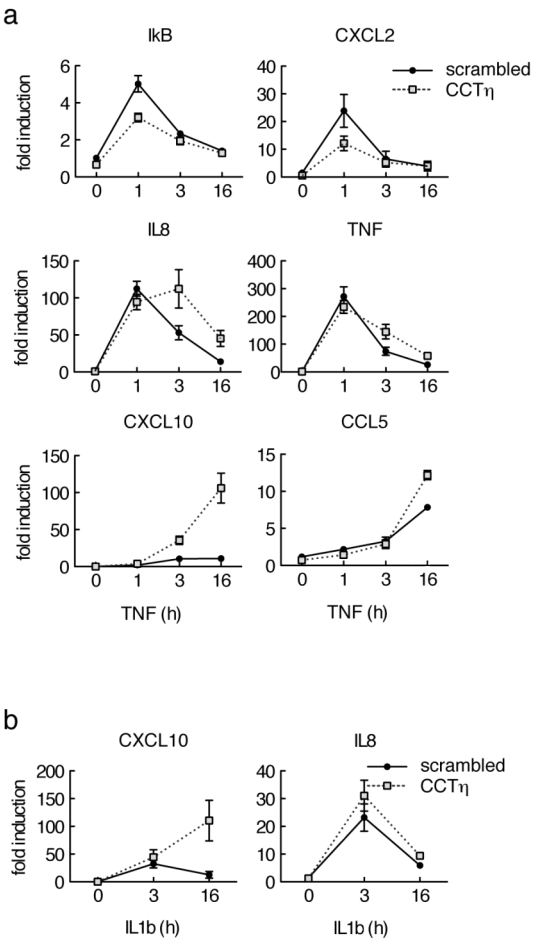

Figure 2.2. CCTη modulates TNF and IL-1β-induced gene expression in a promoter specific way. HeLa cells were exposed to CCTη or scrambled siRNA for three days and stimulated with **(a)** TNF (10 ng/ml) or **(b)** IL-1β (10 ng/ml) for indicated times. mRNA levels were analyzed by qPCR as described under "Materials and Methods". Data represent mean relative mRNA levels ± SEM (n≥3).

Whereas *IκBα* and *CXCL2* mRNA expression was reduced at 1 h after TNF stimulation in CCTη siRNA transfected cells, expression of *TNF*, *IL-8*, *CXCL10* and *CCL5* mRNA increased at 3 and/or 16h after TNF stimulation, as compared to scrambled siRNA transfected controls (see Figure 2.2.a). Similarly to TNF stimulation, CCTη knockdown led to an increase of *IL-8* and *CXCL10* mRNA expression 3 and 16 h after IL-1β addition (see Figure 2.2.b). This suggests the involvement of CCTη in initiation as well as termination of NF-κB-driven transcription, revealing a multi-faced role in regulating NF-κB-dependent genes, possibly acting downstream of IKKs.

3. CCTη regulates NF-κB-DNA binding

Proteolytic IκB degradation is required for NF-κB nuclear translocation, binding to the κB enhancer and transcription initiation (175). In addition, NF-κB-dependent IκBα re-synthesis is critical for termination of NF-κB signaling pathway (150, 151). Thus, we investigated whether CCTη modulates IκBα protein expression. CCTη knockdown had no apparent impact on TNF-induced IκBα degradation, as compared to scrambled siRNA transfected cells (see Figure 2.3.a). In accordance, cytoplasmic and nuclear p65 and p50 protein levels were not influenced in TNF-treated cells transfected with CCTη siRNA (see Figure 2.3.), suggesting that CCTη exerts its effect independently of IκBα, presumably after p65 nuclear translocation. Indeed, we found that CCTη knockdown was associated with increased NF-κB binding to DNA κB consensus sequence at 3 h and 16 h after TNF stimulation, while not affecting binding at 30 min and 1 h, as analyzed by electromobility shift assay (EMSA) (see Figure 2.4.a). Resolved DNA protein complexes were identified as p50/p65 heterodimers by specific binding of these complexes by both p50 and p65 specific antibodies (see Figure 2.4.b).

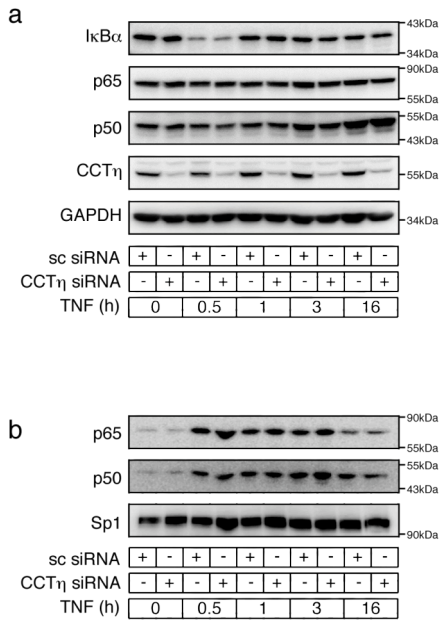

Figure 2.3. CCTη does not influence NF-κB subcellular distribution. HeLa cells were exposed to CCTη or scrambled (sc) siRNA for three days and stimulated with TNF (10 ng/ml) for indicated times. Preparation of cytoplasmic and nuclear extracts was carried out as described under "Materials andMethods". **(a)** IκBα, p65, p50, CCTη and GAPDH were detected in cytoplasmic extracts and **(b)** p65, p50 and Sp1 were detected in nuclear extracts by western blotting. Immunoblots are representative of three independent experiments.

Composition of NF-κB heterodimers was not changed at different time points after TNF stimulation or by CCTη knockdown (see Figure 2.4.b), indicating that CCTη does not affect NF-κB dimer formation. This suggests that increased DNA binding at 3 and 16 h after TNF stimulation could account for increased expression of NF-κB target genes at these time points in CCTη siRNA transfected cells. Impaired gene expression

at 1 h after TNF, on the other hand, cannot be explained by altered DNA binding. Several post-translational modifications that have been shown to modulate NF-κB transcriptional activity without affecting its DNA binding (176) could account for this effect.

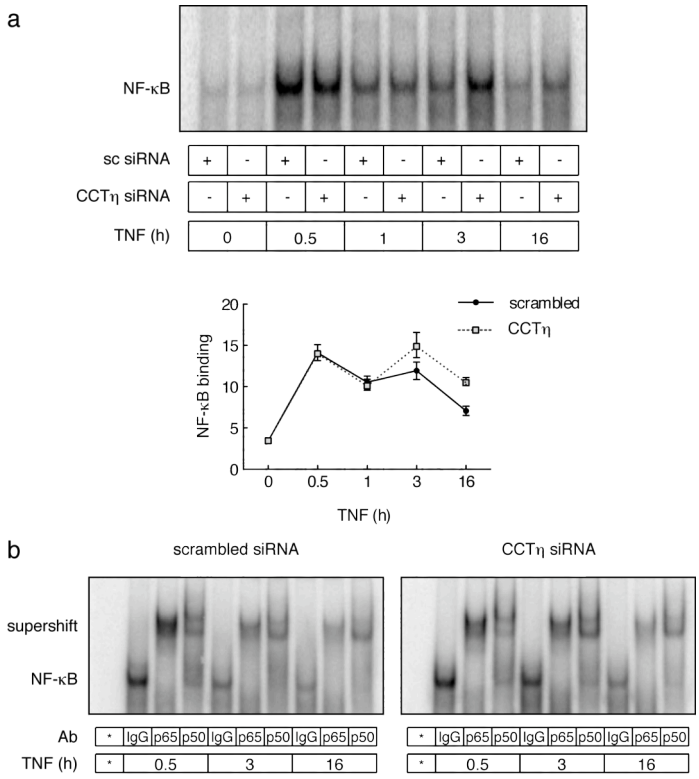

Figure 2.4. CCTη increases NF-κB-DNA binding. HeLa cells were exposed to CCTη or scrambled (sc) siRNA for three days and stimulated with TNF (10 ng/ml) for indicated times. **(a)** Nuclear extracts were analyzed by EMSA using a κB consensus dsDNA oligonucleotide. Data shown represent mean intensity ± SEM (n=3). **(b)** Supershift analysis of NF-κB complexes composed of p65/p50 hetero-dimers representative of two independent experiments. Ab, antibody; *, free probe.

4. CCTη modulates p65 transcriptional activity via a mechanism that targets K122/123 acetylation

Beside *de novo* IκBα expression, p65 acetylation by CBP/p300 can control the "strength" and duration of NF-κB activity. For example, acetylation of p65 K221 enhances its transcriptional activity by increasing DNA binding and impairing its assembly with IκBα (199). Contrary, acetylation at K122 and K123 reduces p65 binding to DNA κB consensus sequence, thus mediating its export from the nucleus (198). HDAC3 can antagonize the effect of CBP/p300 by deacetylation of p65 K122, K123 and K221 (198, 199). Given that the chaperonin CCT is required for HDAC3 activity (253) we hypothesized that CCTη regulates NF-κB DNA binding via modulation of HDAC3-dependent p65 deacetylation. If this were the case then depletion of CCTη should increase p65 binding to DNA κB consensus sequence supposedly by increasing p65 K221 acetylation (199). To test this hypothesis HEK293 cells were co-transfected with myc-tagged p65, CBP encoding expression vector and either CCTη or non-targeting shRNA constructs and p65 acetylation was assessed by immunoblotting with anti-acetyl lysine antibody. Contrary to what would be expected, CCTη knockdown decreased CBP-induced p65 acetylation (see Figure 2.5.a) ruling out HDAC3 as a target of CCTη-mediated effect on NF-κB activity in this experimental system. We next tested whether reduced CBP activity could account for the decreased p65 acetylation in CCTη-depleted cells. As the hypoacetylated form of CBP/p300 has reduced catalytic activity (271), we assessed CBP acetylation status in CCTη depleted cells. CCTη knockdown did not alter CBP protein levels, but reduced CBP acetylation when compared to scrambled shRNA transfected control (see Figure 2.5.b). This suggests that CCTη regulates NF-κB transcriptional activity via a mechanism involving the modulation of p65

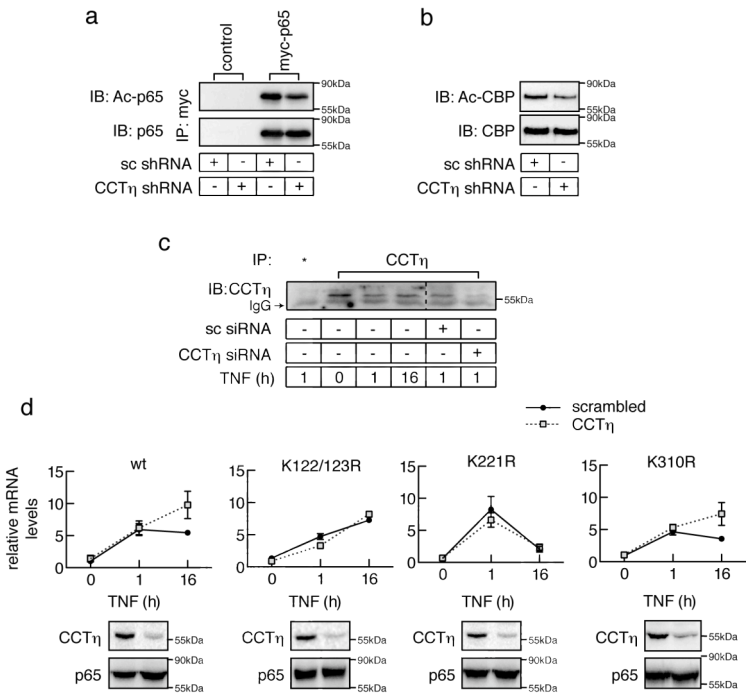

Figure 2.5. CCTη regulates p65 transcriptional activity by modulating CBP-dependent p65 acetylation of lysines 122 and 123. (a) HEK293 cells were transfected with CBP, pCDNA3 (control) or myc-p65 and scrambled (sc) or CCTη shRNA, and p65 was immunoprecipitated using c-myc-specific agarose beads. Acetylated p65 (Ac-p65) and total p65 were detected using antibodies to acetylated lysine or p65, respectively. **(b)** HEK293 cells were transiently transfected with scrambled (sc) or CCTη shRNA, and endogenous acetylated CBP (Ac-CBP) and total CBP were detected using antibodies to acetylated lysine or CBP after immunoprecipitation, respectively. **(c)** HEK293 cells were transfected or not with scrambled (sc) or CCTη siRNA, stimulated with TNF (10ng/ml) for indicated times and nuclear extracts were prepared. CCTη was immunoprecipitated using TCP-1η antibody. Unrelated EP-1 antibody was used as a goat IgG control (*). **(d)** *Rela*–/– MEF reconstituted with either p65 wt, K122/123R, K221R or K310R mutant were exposed to scrambled or CCTη siRNA and stimulated with TNF (10 ng/ml) for indicated times. *Cxcl10* mRNA levels were analyzed by qPCR as described under "Materials and Methods". Data represent mean relative mRNA levels ± SEM (n≥3).

acetylation, presumably by CBP/p300.

While CCT proteins were predominantly described as cytoplasmic, their nuclear localization and function has recently been reported as well (272-275). Since p65 acetylation is thought to be a nuclear event (176) and given that CBP/p300 is expressed in the nucleus (276-278) we assessed whether chaperonin CCTη was expressed in the nucleus as well. CCTη was detected in the nucleus of HEK293 cells stimulated with TNF after immunoprecipitation (see Figure 2.5.c). Specificity was confirmed by lack of nuclear CCTη in CCTη siRNA-transfected cells (see Figure 2.5.c).

Given that CBP/p300 can acetylate p65 on multiple lysines, we assessed whether the effect of CCTη on NF-κB-dependent gene regulation occurs through acetylation on specific K residues. We compared TNF-induced *Cxcl10* mRNA expression in $p65^{-/-}$ MEF reconstituted with wild type p65, K122/123R, K221R or K310R p65 mutants, expressing or not CCTη. All MEF lines showed comparable *Cxcl10* mRNA levels, as assessed 1 h after TNF stimulation (see Figure 2.5.d). CCTη knockdown in MEF expressing wild type p65 increased *Cxcl10* expression at 16 h after TNF stimulation, as compared to scrambled siRNA transfected controls (see Figure 2.5.d). Similar increase was observed in MEF expressing the p65 K310R mutant (see Figure 2.5.d). K221 mutation abolished p65 transcriptional activity at 16 h after TNF stimulation (see Figure 2.5.d), possibly by enhancing IκBα binding and leading to p65 nuclear export, as previously reported (199). CCTη knockdown did not restore the transcriptional activity of this p65 mutant (see Figure 2.5.d). In contrast to the p65 K221 mutant and in agreement with the study published by Kiernan et al. (198), the K122/123R mutation enhanced p65 transcriptional activity 16 h after TNF stimulation, as compared to MEF expressing wild type p65 (see

Figure 2.5.d). Inhibition of CCTη failed to modulate the transcriptional activity of this p65 mutant, suggesting that CCTη facilitates p65 acetylation on K122 and/or K123, reducing p65 DNA binding and gene transcription, implicating CCTη in the termination of NF-κB activity.

IV. Discussion

CCT is a ubiquitously expressed multimer protein complex involved in protein folding as well as assembly of protein complexes in an ATP-dependent manner. Though originally described as a cytosolic protein involved in actin and tubulin folding (246, 247, 279), CCT is also expressed in the nucleus (272-275) (see Figure 2.5.c). CCT can interact with a range of proteins, including von Hippel-Lin (VHL)-elongin BC tumor suppressor complex (249), cell-division cycle protein 20 (Cdc20) (250), sphingosine kinase 1 (251), Polo-like kinase 1 (252), HDAC3 (253), huntingtin (254), as among others (248). This suggests the involvement of CCT in various cellular functions beyond cytoskeleton organization, such as cell cycle, transcription, chromatin remodeling and protein degradation.

CCT is composed of eight different subunits (CCTα-θ) that share similar domains and amino acid sequences conserved across species (243-245). Here we identify the chaperonin CCT subunit η (CCTη) as an evolutionary conserved regulator of NF-κB-dependent transcription that exhibits its effect via a mechanism that targets p65 acetylation. While we have focused specifically on the CCTη subunit, it is likely that a functional chaperonin complex is needed to regulate NF-κB transcription. This notion is supported by the observation that knock-down of the CCT subunits α or ζ had comparable effects to that of CCTη knockdown in regulating TNF-induced reporter activity. Moreover, others

have also shown that knockdown of individual CCT subunits reduces the expression and activity of the CCT chaperonin complex (74, 280).

We have established that CCTη regulates NF-κB transcriptional activity, presumably by targeting p65 after its nuclear translocation. CCTη knockdown modulated both TNF- and IL-1β-driven NF-κB activation (see Figure 2.2.), suggesting that CCTη acts downstream of IKK activation, a common denominator of the signal transduction pathways triggered by cross-linking of the TNF and IL-1 receptors (270). In keeping with this notion, CCTη failed to modulate IκBα degradation as well as p65 nuclear translocation (see Figure 2.3.). Consecutively, we found that CCTη knockdown increased NF-κB binding to DNA κB consensus sequence (see Figure 2.4.), presumably by altering p65 acetylation.

In *Drosophila* cells, Tcp-1η knockdown reduces NF-κB-driven reporter activity, which contrasts to mammalian cells in which CCTη knockdown promotes NF-κB activity in response to TNF (see Figures 2.1.c, 2.1.d). This apparent discrepancy could result from opposing functional outcome of Rel acetylation in Drosophila *vs.* mammalian cells. Whereas Dorsal and Dif acetylation have not been previously shown to regulate their transcriptional activity, p65 acetylation affects NF-κB-dependent transcription in different ways. While K310, K314 and K315 acetylation enhances p65 and hence NF-κB transcriptional activity without affecting DNA binding (199, 200)), K218 and K221 acetylation increases NF-κB transcriptional activity by inhibiting its removal from DNA by newly synthesized IκBα and termination of the transcriptional response (199). In contrast, K122 and K123 acetylation reduces p65 transcriptional activity by decreasing DNA binding in an IκBα-independent manner (198). Hence, the impact of p65 acetylation may be dependent on the promoter context as well as kinetics of the

transcriptional event. Supporting this notion, CCTη knockdown in mammalian cells decreased *IκBα* and *CXCL2* expression, as assessed 1 h after TNF stimulation while increasing that of *TNF*, *IL8*, *CCL5* and *CXCL10*, as assessed 3 and/or 16 h thereafter (see Figure 2.2.a), suggesting a different impact of acetylation on early- and late-phase of NF-κB transcriptional activity.

p65 acetylation is controlled via opposing effects of HATs and HDACs (176). Chaperonin CCT is required for the formation of an enzymatically active HDAC3–SMRT complex (253) that can regulate late phase NF-κB activity by reducing p65 DNA binding, leading to termination of NF-κB dependent transcription (199). This is consistent with the observation that SMRT and CCT co-localize to the promoter region of NF-κB dependent genes (281). Based on these findings, one would expect CCTη knockdown to enhance p65 DNA binding by increasing p65 acetylation, presumably by decreasing the amount of functionally active HDAC3. This is, however, not the case, as CCTη knockdown decreased p65 acetylation (see Figure 2.5.a).

HAT CBP/p300 is a major p65 acetyltransferase (198-200), whose catalytic activity is regulated by a multiplicity of factors (282), including auto-acetylation (271). We found that CCTη knockdown reduced acetylation of endogenous CBP (see Figure 2.5.b), pointing at reduced HAT activity possibly accounting for diminished p65 acetylation. Additional studies will be necessary to address the exact mechanism by which CCTη regulates CBP activity. One possibility is the involvement of cyclin E/cyclin-dependent kinase 2 (Cdk2), which phosphorylates CBP thereby increasing its HAT activity (283). The chaperonin CCT is essential for cyclin E maturation and cyclin E/Cdk2 complex formation in yeast and CCTβ/cyclin E association has been shown in mammalian cells (255).

Regulation of p65 activity by acetylation is target residue dependent. While K310 deacetylation diminishes p65 transcription without affecting DNA binding, decreased K221 acetylation favors IκBα binding, promoting its removal from DNA and nuclear export (199). It is therefore unlikely that CCTη modulates acetylation of these residues during late phase of NF-κB activation. Consistent with this, CCTη knockdown increased transcriptional activity of p65 K310R mutant 16 h after TNF stimulation in a similar manner, as observed in MEF expressing wild type p65 (see Figure 2.5.d). On the other hand, the p65 K221R mutant showed significantly lower transcriptional activity, as assessed 16 h after TNF stimulation (see Figure 2.5.d), possibly reflecting enhanced nuclear export after *de novo* IκBα synthesis (199), and was therefore insensitive to CCTη knockdown (see Figure 2.5.d). We cannot, however, exclude that CCTη knockdown down-regulates K310 acetylation at earlier –time points, which could explain transcriptional repression of *IκBα* and *CXCL2* genes as assessed 1 h after TNF stimulation (see Figure 2.2.a).

Acetylation of p65 K122 and K123 inhibits DNA binding, promoting termination of NF-κB-dependent transcriptional response (198). In line with this, the p65 K122/123R mutant failed to terminate NF-κB-dependent transcription reflected by increased *Cxcl10* mRNA, as compared to p65 wt or p65 K310R and K221R mutants (see Figure 2.5.d). Interestingly, the kinetics of mRNA expression of this mutant was similar to that observed in CCTη depleted cells expressing wild type p65, and was insensitive to CCTη knockdown (see Figure 2.5.d). This suggests that CCTη regulates NF-κB-dependent transcription via K122 and K123 acetylation.

In conclusion this study identifies the chaperonin subunit CCTη as a negative regulator of NF-κB transcriptional activity via a mechanism

involving the modulation of p65 acetylation, presumably by altering CBP activity. We propose that CCT chaperonin might be involved in terminating NF-κB signaling and as such may be important for the resolution of inflammation.

V. Materials and Methods

Cell Culture

Drosophila S2 cells were kindly provided by Monica Bettencourt Dias (Instituto Gulbenkian de Ciência, Oeiras, Portugal) and were grown at 25°C in Schneider's Drosophila medium (Invitrogen). HeLa and human embryonic kidney 293 (HEK293) cells were obtained from ATCC. Mouse embryonic fibroblasts (MEF) isolated from *Rela*$^{-/-}$ mice were kindly provided by Dr. Amer Beg (Moffitt Cancer Center, Tampa, FL) and have been previously described (108). *Rela*$^{-/-}$ MEF were stably transfected with human RelA wt, K122/123R, K221R and K310R mutant recombinant retroviruses essentially as described (219). Cells were grown at 37°C in Dulbecco's modified Eagle's medium (MediaTech) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 μg/ml streptomycin B (all Atlanta Biologicals) in a humidified atmosphere containing 5% CO_2.

Plasmid Constructs

Drosomycin-luciferase (*Drs-luc*) and Toll 10b expression vectors were kindly provided by Dr. Mika Ramet (University of Tampere, Tampere, Finland) and were described elsewhere (135). Dorsal and Dif were amplified from the original pAct-dl and pAct-Dif vectors (kind gift from Dr. Ylva Engstrom, Stockholm University, Stockholm, Sweden (268)) by PCR (Dorsal 5'-GAT CCT CGA GAT GTT TCC GAA CCA GAA CAA TGG AG-3' and 5'-TCG ATC TAG ACG TGG ATA TGG ACA GGT TC-

3'; Dif 5'-GAT CCT CGA GAT GTT TGA GGA GGC TTT C-3' and 5'-TCG ATC TAG ATT TGA ATG GCT GAA TTC CCA AG-3´) and cloned (*Eco*RV/*Xba*I) and (*Xho*I/*Xba*I) into pAc5.1/V5-HisA vector (Invitrogen), respectively. NF-κB luciferase reporter construct, i.e. *κB-luc*, has been described elsewhere (284). Scrambled and CCTη shRNA constructs were cloned by inserting annealed oligonucleotides containing a 5'-GAG TGT TTG AGT TTG AGA TCC-3' or a unique 19bp sequence derived from the mRNA transcript of CCTη gene (5'-GCC ACA AAC ATT CTC AAC A-3') between the unique *Bgl*II and *Hind*III enzyme sites of the pSUPER vector (OligoEngine). N-terminal myc-tagged p65 expression vector has been described (285). C-terminal HA-tagged CBP was expressed from a pcDNA3 vector. p65 K122/123R, K221R and K310R expression vectors were generated by cloning a p65 sequence containing K122/123R, K221R and K310R mutation, respectively, into a pLXIH vector as described (219). All constructs were verified by automated DNA sequencing using dye termination chemistry.

Transient transfection and reporter assay

S2 cells were cultured in 12-well plates ($5x10^5$ cells per well) and a day after exposed to 1 μg of total DNA (150 ng *Drs-luc*, 5 ng Toll 10b, 50 ng Dorsal, 50 ng Dif) and 5 μl of Cellfectin transfection reagent according to manufacturer's instructions (Invitrogen) in Drosophila SFM medium (Invitrogen) for 12 h at 25°C. Cells were washed and transferred into 96-well plates containing lacZ dsRNA (900ng per $5x10^4$ cells). After incubation for three days, firefly (Luc) and Renilla (Ren) luciferase activity were measured using Dual-luciferase Reporter Assay System (Promega). HEK293 cells were grown in 12-well plates and transfected at 90% confluency. Cells were exposed to 1 μg of total DNA (300 ng of reporter plasmid, 500 ng of shRNA plasmid and 40 ng of

cytomegalovirus enhancer/β-galactosidase control plasmid) and 1 μl of Lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen) in Dulbecco's modified Eagle's medium for 3 h. After addition of fetal bovine serum to a final concentration of 10%, cells were incubated (three days, 37°C), stimulated with TNF (10 ng/ml, 6 h), lysed with passive lysis buffer (Promega) and supernatants were assayed for luciferase and β-galactosidase activity as described elsewhere (284).

RNA interference, transient transfection and reporter assay

S2 cells were cultured in 12-well plates and transfected as above (150 ng *Drs-luc* reporter plasmid, 5 ng Toll 10b plasmid). Cells were washed, diluted to $1x10^6$ cells/ml and a total of 10 μl of cells were added to the dsRNA-containing 384-well plate (250 ng dsRNA/well), resulting in a final concentration of $1x10^4$ cells per well. Plates were gently centrifuged, cells were incubated (30 min, room temperature) and 30 μl of Schneider's medium containing 15% fetal bovine serum was added. Plates were sealed to prevent evaporation and incubated for three days at 25°C. Luc and Ren activity was measured using Dual-luciferase Reporter Assay System according to manufacturer's instruction (Promega). 87 wells not containing dsRNA on each experimental plate were used to calculate the coefficient of variation (CV) for Luc and Ren activities. All three experimental plates showed CV of 7.4±1.7% for Luc and 30.3±14.5% for Ren. Because of the aberrant Ren activity in our experimental system, possibly due to the affected Actin promoter-driven expression by certain dsRNA (286), Luc values could not be normalized to Ren values. The positive control in screen and data processing, dsRNA of DIAP1 inhibitor of apoptosis, led to a very significant reduction of Luc signals in two out of three experimental plates that were considered for further analysis. Experimental data were normalized by

setting the average Luc value of wells not containing dsRNA to 1. Values lying outside the mean ± 2SD boundaries in two experiments were considered indicative for putative candidate genes.

dsRNA synthesis and screen confirmation

dsRNAs targeting putative genes identified in the screen were generated by *in vitro* transcription of a PCR-generated DNA template (RNAi probes, FlyBase) containing the T7 promoter sequence on both ends (RiboMAXTM Large Scale RNA Production System-T7, Promega) and purified using Mini Quick Spin RNA Columns (Roche). Transiently transfected S2 cells (150 ng *Drs-luc*, 50 ng Dorsal, 50 ng Dif, 5 ng Toll 10b per $5x10^5$ cells) were exposed to dsRNA (900 ng per $5x10^4$ cells) in 96-well plate, incubated for three days and Luc and Ren activity was measured using Dual-luciferase Reporter Assay System (Promega).

CCTη knockdown by siRNA

siRNA knockdown of CCTη was performed in a 12-well plate format in HeLa ($6x10^4$ cells per well), HEK293 ($3x10^5$ cells per well) and MEF ($5x10^4$ cells per well) cells. Cells were exposed to 50 nM siGENOME Non-Targeting siRNA #1 or CCTη siRNA and 1 μl of DharmaFECT1 transfection reagent according to manufacturer's instructions (Thermo Scientific). CCTη siRNA target sequences were human: 5'-GCC ACA AAC ATT CTC AAC A-3' and mouse: 5'- GCC ACA AAC ATC CTC AAC A -3'.

Gene expression analysis

Real-time quantitative PCR (qPCR) was carried out using SYBR Green chemistry (Invitrogen) on a Chromo4 continuous fluorescence monitoring thermocycler (MJ Research) as described previously (219). Relative

transcript levels were determined by normalization to the housekeeping gene *hypoxanthine guanine phosphoribosyl transferase* (*HPRT*). Primers used for expression analysis in HeLa cells were: *IκBα* (5'-TCC TGT TGA AGT GTG GGG CTG ATG-3' and 5'-CCT CCA AAC ACA CAG TCA TCA T-3'), *CXCL2* (5'-CAC TCA AGA ATG GGC AGA AAG-3' and 5'-TCA GGA ACA GCC ACC AAT AAG-3'), *IL8* (5'-TCC TGA TTT CTG CAG CTC TGT-3' and 5'- TGT GGT CCA CTC TCA ATC ACT C-3'), *TNF* (5'-AGT GCT GGC AAC CAC TAA GAA-3' and 5'-ATT CCA GAT GTC AGG GAT CAA-3'), *CXCL10* (5'-CCT CTC CCA TCA CTT CCC TAC-3' and 5'-GCT GAT TTG GTG ACC ATC ATT-3'), *CCL5* (5'-TGC CCA CAT CAA GGA GTA TTT-3' and 5'-CCA TCC TAG CTC ATC TCC AAA-3') and *HPRT* (5'-TTC TGT GGC CAT CTG CTT AGT-3' and 5'-GCC CAA AGG GAA CTG ATA GTC-3'). Primers used for expression analysis in MEF were: *Cxcl10* (5'-AAG TCA GCC AAT CAG GAC TCA-3' and 5'-GTT GGC TCG GGA TGT CTC T-3'). Primers for the mouse *Hprt* have been described elsewhere (219). Expression levels of unstimulated, scrambled siRNA-exposed cells were set to 1, and fold induction for other experimental groups was calculated. *CXCL10* mRNA levels of TNF-unstimulated HeLa cells were not consistently detectable; expression level of *CXCL10* mRNA in CCTη siRNA-exposed cells 16 h after TNF was set to 100 and relative values for other experimental groups were calculated.

Preparation of subcellular extracts, EMSA and western blotting

HeLa cells were transfected with siRNA as described above and incubated for three days before stimulation with TNF (10 ng/ml). Preparation of cytoplasmic/nuclear extracts and EMSA were carried out as described previously (285). Briefly, cells were lysed in hypotonic buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% TritonX-100, supplemented with protease inhibitors) and centrifuged (600 *g*, 5

min, 4°C). Supernatant (cytoplasmic fraction) was transferred into new tubes and the remaining pellet (nuclear fraction) was washed once in hypotonic buffer and lysed (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 20-800 mM NaCl, 0.2 mM EDTA, 1 mM PMSF, 1 mM DTT, proteasome inhibitors). Nuclear extracts were incubated with 100,000 cpm of double-stranded [γ-^{32}P]ATP-radiolabled NF-κB oligonucleotide (5'-AGT TGA GGG ACT TTC CCA GGC-3'; 30 min, room temperature; PerkinElmer) and the resulting DNA-protein complexes were separated on a 6% polyacrylamide gel in Tris/glycine/EDTA buffer pH 8.5. Experimental data were analyzed by setting the net intensity of EMSA bands to 100 and calculating the percentage of intensity for each data point. For supershift analysis, labeled DNA-protein complexes were incubated with 1 μg of p65 (sc-8008x, Santa Cruz) or p50 (sc-114x, Santa Cruz) specific antibodies or non-immune rabbit IgG (I5006, Sigma) (1 h, 4°C) prior to separation. For western blotting, proteins contained in cytoplasmic and nuclear fractions were resolved by electrophoresis on 10% SDS-polyacrylamide gels, transferred to polyvinylidene fluoride membranes and detected with antibodies against IκBα (IMG-127A, Imgenex), p65 (sc-372, Santa Cruz), p50 (sc-7178, Santa Cruz), CCTη (sc-13889, Santa Cruz), GAPDH (MAB374, Millipore) and Sp1 (sc-59, Santa Cruz). Primary antibodies were detected using HRP-conjugated secondary antibodies (Santa Cruz).

Immunoprecipitation and protein acetylation

HEK293 cells were grown in 12-well plates and transfected with calcium phosphate. Cells were exposed to 1.6 μg of total DNA (50 ng of myc-tagged p65, 200 ng of HA-tagged CBP expressing construct and 1 μg of shRNA plasmid), incubated for three days and stimulated with TNF (10 ng/ml, 30 min). Cells were lysed (50 mM HEPES pH 7.9, 250 mM NaCl,

1% NP-40, 1 mM EDTA, protease inhibitors) and centrifuged (10 min, 16000 *g*, 4°C). Supernatants were incubated with 15 μl c-myc-conjugated agarose beads (2 h, 4°C, Sigma). For CBP detection, cells were transfected with CCTη or scrambled shRNA, stimulated with TNF (10 ng/ml, 30 min) and cell extracts were incubated with 1 μg CBP antibody (o/n, 4°C, sc-369, Santa Cruz) and 20 μl Protein-A Sepharose beads (1 h, 4°C, GE Healthcare). For CCTη detection, HEK293 cells were exposed or not to CCTη or scrambled siRNA and stimulated with TNF (10 ng/ml) for indicated times. Nuclear extracts were incubated with 1 μg TCP-1η antibody (sc-13889, Santa Cruz) and 20 μl Protein-A Sepharose beads. Precipitated proteins were washed in lysis buffer, resuspended in 2x SDS sample buffer, resolved by SDS-PAGE and subjected to western blotting with antibodies detecting acetylated lysine (9814S, Cell Signaling), p65 (sc-372, Santa Cruz), CBP or CCTη. Primary antibodies were detected using Protein-A-HRP conjugates (Millipore) or respective secondary antibodies. Purity of nuclear extracts was confirmed using GAPDH antibody (MAB374, Millipore).

VI. Author contribution and Acknowledgments

Author contribution: **NP** designed and performed all experiments, analyzed data and was assisted by KH. TL and BLA generated the plated *Drosophila* dsRNA used in the gene-screening assay. MPS and JA designed the study. The manuscript was written by **NP** with assistance from JA and MPS.

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CHAPTER III
REGULATION OF NF-κB ACTIVITY BY SUBNUCLEAR LOCALIZATION

I. Summary

RelA is a member of the nuclear factor kappa B (NF-κB) family of transcription factors regulating inflammation as well as cell growth and programmed cell death (PCD). Distinct mechanisms have evolved to control NF-κB activity. The central one relies on the inhibitory effect exerted by inhibitors of κB (IκB) molecules that bind transcriptionally active NF-κB family members such as RelA, retaining them in the cytoplasm. Proteolytic degradation of IκB is required for NF-κB nuclear translocation and initiation of transcription. RelA translocation into specific nuclear bodies adds an additional layer of control over NF-κB-dependent gene transcription, a mechanism regulated by post-translational modifications, including RelA phosphorylation and ubiquitination. RelA serine (S) 276 phosphorylation controls NF-κB transcriptional activity by promoting its interaction with cofactors of transcriptional machinery, as well as by inhibiting RelA ubiquitination and subsequent proteasomal degradation. Here we provide evidence that RelA S276 phosphorylation also regulates its translocation into promyelocytic leukemia protein (PML) nuclear bodies. We show that the hypophosphorylated RelA S276A mutant translocates preferentially to PML nuclear bodies via a mechanism that depends on RelA DNA binding. This occurs presumably via the activation of protein kinase A (PKA).

II. Background

The mammalian nuclear factor kappa B (NF-κB)/Rel family of transcription factors (TF) is composed of homo- or heterodimeric complexes resulting from the interaction of five members, i.e. p65/RelA,

RelB, c-Rel, p105/p50 and p100/p52. These share within their N-terminal region a Rel Homology Domain (RHD), responsible for dimer formation, interaction with IκB, nuclear translocation and DNA binding (86). RelA, RelB and c-Rel also contain a C-terminal transactivation domain (TAD), defining these members transcriptionally active (86). Among these, RelA plays a predominant role in the regulation of so called proinflammatory genes associated with stress responses to injury and/or microbial infection (140). While NF-κB activation is strictly required to trigger inflammation, uncontrolled NF-κB activity can be associated with inability to resolve inflammation leading to tissue damage. Presumably, this contributes in a critical manner to the pathogenesis of immune-mediated inflammatory diseases, in which inflammation is underlying cause of disease. (84)

Under homeostasis, transcriptionally active NF-κB dimers are retained in the cytoplasm by inhibitory proteins of the IκB family (89). p100 and p105, on the other hand, contain IκB-like ankyrin repeats within their C-terminal region, sequestering these proteins in the cytoplasm (86). Proteolytic degradation of IκB is required for NF-κB nuclear translocation and binding to the κB consensus sites within the promoter regions of NF-κB-dependent genes, where dimer composition can confer some level of specificity towards different κB sites (287). One of these NF-κB-dependent genes is IκBα, which interacts with the promoter-bound RelA leading to its nuclear export, a feedback loop that contributes to termination of NF-κB transcriptional response (150, 151).

The specificity of NF-κB transcriptional response is achieved by numerous post-translational modifications, including some that target directly Rel proteins (175, 176). RelA phosphorylation at S276 induces its conformational changes, regulating its transcriptional activity by modulating the interaction of RelA with other proteins. These include

transcriptional co-activators, e.g. CREB-binding protein (CBP)/p300 (181), positive transcription elongation factor b (P-TEFb) (288), as well as transcriptional repressor histone deacetylase 1 (HDAC1) (182). RelA S276 phosphorylation can also inhibit RelA ubiquitination and degradation via a mechanism that inhibits binding of the E3 ubiquitin ligase suppressor of cytokine signaling 1 (SOCS-1) (289).

Nuclear bodies are a heterogeneous group of dynamic subnuclear structures, implicated in the regulation of gene transcription (232). Nucleoli, the most prominent of all nuclear bodies, are associated with the genes encoding 35S ribosomal RNA, regulating their transcription (290). Moreover, nucleoli can also sequestrate several cell-cycle checkpoint control proteins, e.g. murine double minute 2 (Mdm2) (291), cell division cycle protein 14 (Cdc14) (292) and tumor suppressor proteins, e.g. retinoblastoma protein 1 (pRb1) (293), suggesting an important role of nucleoli in cellular proliferation and cellular stress responses. Along this, nucleolar RelA sequestration can also regulate NF-κB-driven gene transcription (233).

Other nuclear bodies, e.g. nuclear speckles and PML nuclear bodies, are also involved in transcriptional regulation. While not serving as sites of active transcription, speckles are enriched in pre-messenger (m)RNA splicing machinery necessary for pre-mRNA processing (230). In addition, a subset of transcription factors can localize to nuclear speckles, suggesting their regulatory role in transcription (230).

The physiological relevance of PML nuclear bodies was first suggested in studies with acute promyelocytic leukemia patients (294, 295). The identification of proteins associated with PML bodies provided clues to the function of these subnuclear compartments implicating PML bodies in the transcriptional regulation. This can occur via the physical association of PML bodies with the transcriptionally active gene loci, e.g. major histocompatibility (MHC) class I gene cluster (296), by

sequestration of transcriptional cofactors, e.g. CBP (236), or by the induction of TF post-translational modifications, e.g. p53 phosphorylation (297, 298) and acetylation (299). RelA translocation into PML nuclear bodies can also down-regulate NF-κB-dependent transcription via a mechanism involving RelA ubiquitination by a nuclear protein containing postsynaptic density 65-disc large-zonula occludens 1 (PDZ) and abnormal cell lineage 11-islet 1-mechanosensory abnormal 3 (LIM) domains (PDLIM2) (211). Several other proteins involved in NF-κB-dependent transcription, e.g. CBP/p300, IKKε, can also be sequestered in PML nuclear bodies (190, 236), suggesting that PML nuclear bodies may enable the fine control of NF-κB transcriptional activity via a mechanism involving post-translational modifications. Here we present evidences that hypophosphorylated RelA S276A mutant translocates to PML nuclear bodies, an event possibly associated with its gene-specific transcriptional activity.

III. Results

In the experiments presented below, we have co-expressed N-terminal EGFP-tagged wild type RelA (EGFP-RelA), used as a reporter protein, together with different N-terminal myc-tagged RelA variants as effector proteins in ~7-times molar excess (HEK293FT cells) or ~3.5-times molar excess (COS7 cells). Because of the high molar ratio of effector *versus* reporter protein, the premise is that the EGFP-RelA reporter is expressed primarily as a heterodimer with the different myc-tagged RelA effector proteins. This is supported by our observation that co-expression of different myc-tagged RelA effector proteins with EGFP-RelA reporter protein imposes localization of reporter-effector heterodimers. The co-expression of EGFP-RelA reporter protein with

different effector proteins, allows the visualization of the heterodimers while presumably not influencing their localization. We will therefore, for the simplicity reason, refer to different heterodimers only by the name of the effector protein.

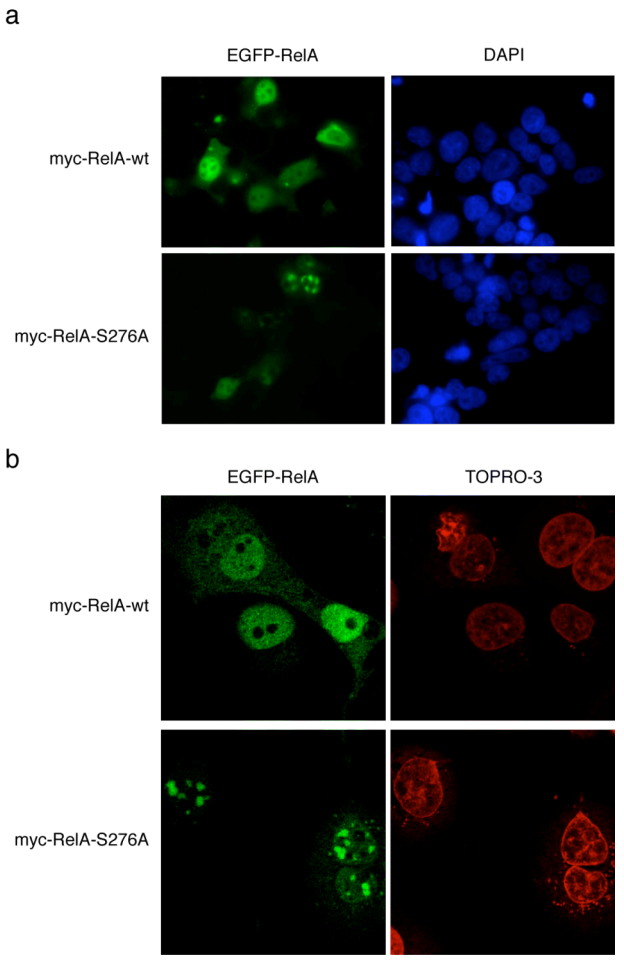


Figure 3.1. Distinct RelA S276A subnuclear localization. (a) HEK293FT or **(b)** COS7 cells were transiently co-transfected with EGFP-tagged RelA (EGFP-RelA) expression construct together with RelA wild type (myc-RelA-wt) or RelA-S276A mutant (myc-RelA-S276A). Cells were fixed and subcellular distribution of EGFP-RelA (green) and DNA (blue or red) was analyzed by fluorescent microscopy.

1. Subnuclear localization of the RelA S276A mutant

RelA S276 phosphorylation is essential to sustain NF-κB transcriptional activity (182, 288, 289). RelA translocation into distinct nuclear compartments also regulates its transcriptional activity (233). To assess whether S276 phosphorylation influences RelA subcellular localization, the fine nuclear localization of RelA was assessed in HEK293FT cells transiently co-transfected with EGFP-RelA together with hypophosphorylated form of RelA, mimicked by the RelA S276A mutation, (myc-RelA-S276A) versus control HEK293FT cells, transiently co-transfected with the wild type RelA (myc-RelA-wt). While the RelA-wt had a homogenous nuclear localization (see Figure 3.1.a), RelA-S276A translocated into distinct nuclear compartments (see Figure 3.1.a). A similar nuclear distribution was observed in COS7 cells (see Figure 3.1.b), suggesting that this phenomenon is not cell type-specific.

2. Hypophosphorylated RelA S276A mutant translocates to PML nuclear bodies

Sequestration of several transcription factors as well as transcriptional cofactors into distinct nuclear bodies regulates gene expression (232). We assessed whether RelA-S276A would translocate to specific nuclear bodies by labeling cells transiently co-transfected with EGFP-RelA and myc-RelA-S276A with antibodies against fibrillarin, anti-splicing factor sc-35 or PML, marker proteins for nucleolus, nuclear speckles or PML nuclear bodies, respectively. Whereas RelA-S276A was excluded from nucleolus and nuclear speckles, it colocalized with PML nuclear bodies (see Figure 3.2.), implicating PML bodies in the regulation of NF-κB-dependent gene expression via a mechanism that involves RelA S276 phosphorylation.

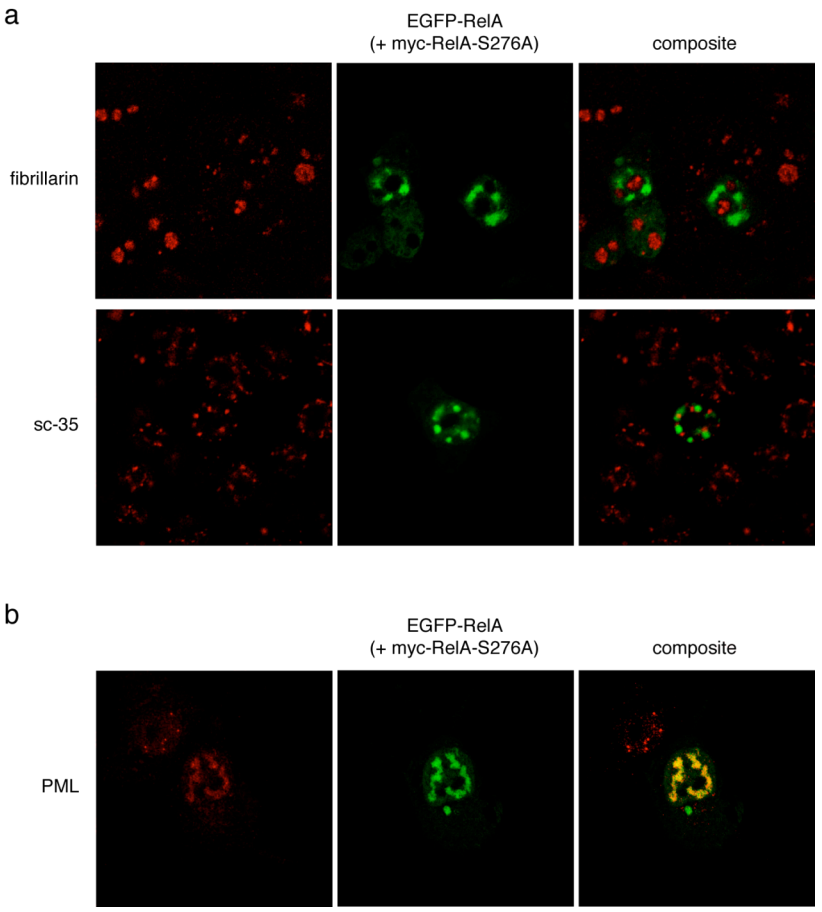


Figure 3.2. RelA S276A mutant translocates to PML nuclear bodies. (a) HEK293FT cells were transiently co-transfected with EGFP-tagged RelA (EGFP-RelA) expression construct together with RelA-S276A mutant (myc-RelA-S276A). Cells were fixed and subnuclear distribution of EGFP-RelA (green), nucleolus (fibrillarin; red) and nuclear speckles (sc-35; red) was analyzed by fluorescent microscopy. **(b)** COS7 cells were transfected as in (a), fixed and subnuclear distribution of EGFP-RelA (green) and PML nuclear bodies (PML; red) was analyzed by fluorescent microscopy.

3. PKA regulates RelA subnuclear localization

RelA S276 can be phosphorylated by PKAc while catalytically inactive form of PKAc, mimicked by K72M point mutation, (DN-PKA) acts as a dominant negative mutant interfering with NF-κB activation, presumably by preventing RelA S276 phosphorylation (300). To assess whether PKAc regulates RelA translocation into distinct nuclear compartments, RelA subnuclear localization was visualized in HEK293FT cells transiently co-transfected with EGFP-RelA and myc-RelA-wt together with the DN-PKA or empty vector as a control. While RelA-wt had

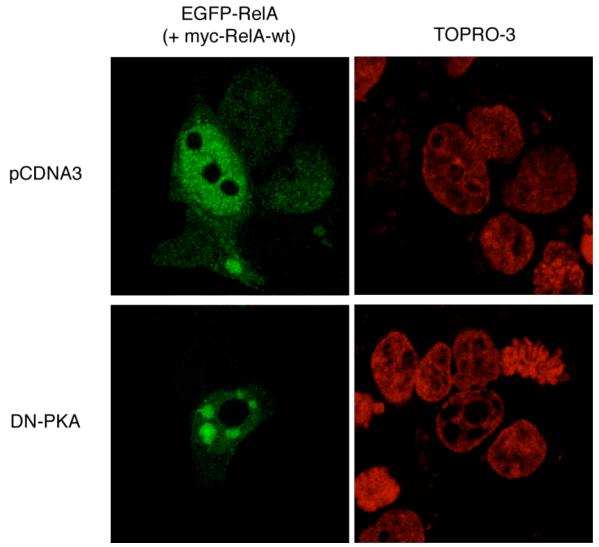


Figure 3.3. PKA activity influences RelA subnuclear localization. HEK293FT cells were transiently co-transfected with EGFP-tagged RelA (EGFP-RelA) expression construct and RelA wild type (myc-RelA-wt) plus catalytically inactive form of PKAc (DN-PKA) or empty vector (PCDNA3) as a control. Cells were fixed and subcellular distribution of EGFP-RelA (green) and DNA (red) was analyzed by fluorescent microscopy.

homogeneous nuclear distribution in control cells, the expression of DN-PKA resulted in its translocation into distinct nuclear compartments (see Figure 3.3.). This suggests that recruitment of RelA to those nuclear compartments is associated with decreased RelA S276 phosphorylation resulting from PKA inhibition.

4. RelA S276A subnuclear localization depends on DNA binding but not on the presence of TAD

RelA transcriptional activity requires the interaction of its TAD with co-activators and components of the basal transcriptional machinery. RelA S276 phosphorylation facilitates these interactions, as illustrated for the transcriptional co-activator CBP/p300 (181) that enhances NF-κB-dependent transcription. RelA S276A mutant, on the other hand, associates preferentially with transcriptional co-repressor HDAC1, reducing NF-κB activity (182, 219). To assess whether RelA transcriptional activity and RelA nuclear distribution are functionally linked, we made use of a construct encoding for the RHD portion of the RelA-wt (myc-RelA-RHD) and lacking TAD, which binds DNA and functions as a dominant negative mutant, repressing NF-κB-dependent transcription (301). RelA subnuclear distribution was monitored in HEK293FT cells transiently co-transfected with EGFP-RelA together with myc-RelA-RHD or myc-RelA-wt as a control. RelA-RHD had homogenous nuclear distribution similarly to control cells co-expressing RelA-wt (see Figure 3.4.a), suggesting that translocation of RelA into distinct nuclear compartments was probably not due to its insufficient transcriptional activity, mimicked by the lach of TAD.

We next asked whether the TAD portion of RelA was responsible for the translocation of RelA S276A to distinct nuclear compartments. RelA subnuclear localization was assessed in HEK293FT cells

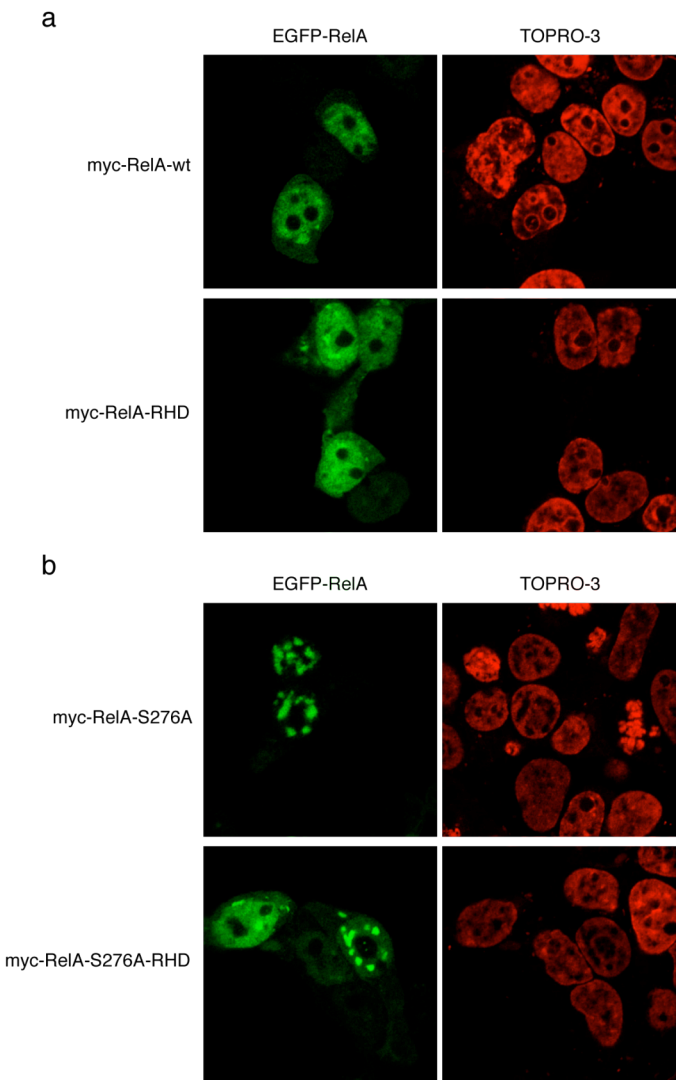


Figure 3.4. RelA subnuclear localization does not depend on its transcriptional activity. HEK293FT cells were transiently co-transfected with EGFP-tagged RelA (EGFP-RelA) expression construct together with **(a)** RelA wild type (myc-RelA-wt) or RelA RHD wild type (myc-RelA-RHD) or **(b)** myc-RelA-S276A or RelA RHD S276A mutant (myc-RelA-S276A-RHD). Cells were fixed and subcellular distribution of EGFP-RelA (green) and DNA (red) was analyzed by fluorescent microscopy.

transiently transfected with EGFP-RelA together with the construct encoding for the RHD portion of the RelA-S276 (myc-RelA-S276-RHD) and lacking TAD or full length myc-RelA-S276A as a control. RelA-S276A translocated into distinct nuclear compartments independently of the presence of TAD (see Figure 3.4.b), indicating that RHD but not the TAD is required for the observed RelA S276A subnuclear localization.

The RelA RHD is required for NF-κB dimer formation, nuclear translocation, DNA binding and interaction with IκB (86). Overexpressed RelA can translocate to the nucleus (see Figure 3.1.), probably

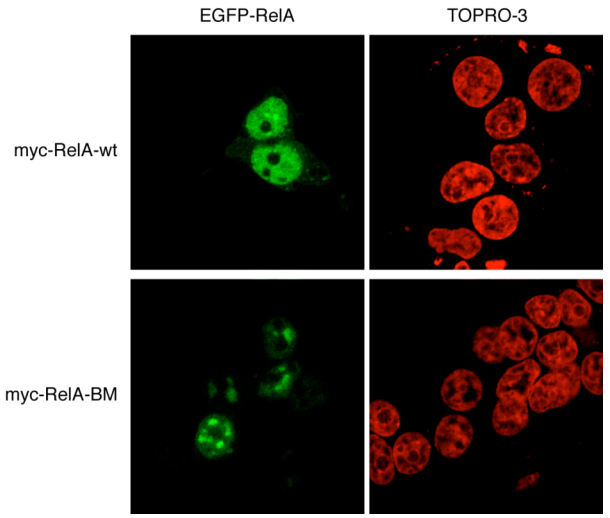
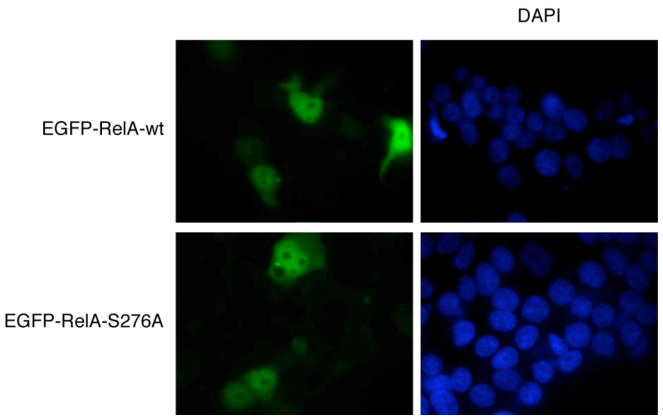


Figure 3.5. RelA-DNA binding influences RelA subnuclear localization. HEK293FT cells were transiently co-transfected with EGFP-tagged RelA (EGFP-RelA) expression construct plus RelA wild type (myc-RelA-wt) or RelA wild type DNA binding deficient mutant (myc-RelA-BM). Cells were fixed and subnuclear distribution of EGFP-RelA (green) and DNA (red) was analyzed by fluorescent microscopy.

overcoming the endogenous mechanisms regulating IκB-dependent RelA cytoplasmic localization. Hypophosphorylated RelA binds DNA with lower affinity as compared to wild type RelA, presumably via a mechanism involving the interaction of RelA N- and C-terminal regions, masking the DNA binding domain within RHD. This mechanism is regulated by PKA and involves phosphorylation of RelA S276 (181). To investigate if DNA binding is important for RelA nuclear distribution, RelA subnuclear localization was monitored in HEK293 cells transfected with EGFP-RelA together with RelA DNA binding-deficient mutant (myc-RelA-BM) or myc-RelA-wt as a control. RelA-BM translocated into distinct nuclear compartments with a pattern similar to that associated with the expression of RelA-S276A, as compared to cells expressing RelA-wt (see Figure 3.5.). This suggests that RelA DNA binding prevents RelA translocation into distinct nuclear compartments.

IV. Discussion

We have co-expressed N-terminal EGFP-tagged wild type RelA (EGFP-RelA) as a reporter protein, together with different myc-tagged RelA variants. This manipulation was necessary because EGFP-tagged RelA-S276A effector protein, i.e. EGFP-RelA-S276A, did not visibly localize into specific subnuclear structures when expressed alone (see Supplementary Figure 3.1.). The reason for this is not entirely clear. One possibility is that the concentrations needed to accumulate enough EGF-RelA-S276A in PML bodies do not permit the visualization of subnuclear structures because of the saturating concentrations of the fluorescent EGFP-tag. An alternative interpretation would be that the N-terminal EGFP tag interferes with RelA trafficking into PML nuclear bodies, a notion in keeping with the different subnuclear localization of N-terminal and C-terminal YFP-tagged RelA (302). Because the EGFP-RelA



Supplementary Figure 3.1. RelA subcellular localization. HEK293FT cells were transiently transfected with EGFP-tagged RelA wild type (EGFP-RelA-wt) or EGFP-tagged RelA S276A mutant (EGFP-RelA-S276A) expression construct. Cells were fixed and subcellular distribution of RelA (green) and DNA (red) was analyzed by fluorescent microscopy.

reporter protein in our experimental system should be preferentially in heterodimeric formation with the myc-tagged effector protein, the translocation of heterodimers into PMLs would not be inhibited.

PML nuclear bodies regulate the availability and activity of transcription factors as well as chromatin-remodeling processes (231, 303). In keeping with this notion, PML nuclear bodies can activate p53 by promoting its acetylation and phosphorylation (299). In addition, several other transcription factors and regulators localize dynamically to PML nuclear bodies. These include CBP/p300 (236, 304), a transcriptional co-activator of NF-κB-dependent transcription and a major RelA acetyltransferase as well as transcriptional co-repressors HDACs (305), which can also deacetylate RelA.

RelA translocation into PML nuclear bodies is controlled by phosphorylation and/or ubiquitination reducing NF-κB-dependent gene expression (190, 211). The mechanisms underlying this phenomenon are not fully understood. We found that RelA translocation to PML nuclear bodies is controlled by S276 phosphorylation. This notion is supported by the observation that hypophosphorylated RelA S276A mutant accumulates into PML nuclear bodies (see Figure 3.2.). The interplay between RelA S276 phosphorylation and its subsequent ubiquitination has been previously identified in that RelA S276 phosphorylation reduces its ubiquitination by SOCS-1 thus increasing the half-life of RelA (289). Therefore, It would be interesting to assess whether subnuclear localization of RelA S276A is dependent on ubiquitination as well as to assess RelA protein stability in PML nuclear bodies.

RelA S276 can be phosphorylated via the activation of at least three protein kinases, namely, PKA (181), mitogen- and stress-activated kinase 1 (MSK1) (185) and Pim-1 (289). While we did not assess the effect of MSK1 and Pim-1 activity on the RelA subnuclear localization, we did so for PKA and found that its activity was necessary to prevent the translocation of RelA to PML nuclear bodies (see Figure 3.3.). Our data also supports the notion that RelA translocation into PML nuclear bodies is dependent on RelA DNA binding, as suggested by the observation that the RelA DNA binding-deficient mutant translocates into PML nuclear bodies (see Figure 3.5.). While it is generally accepted that RelA phosphorylation does not influence DNA binding, hypophosphorylated S276 RelA probably binds DNA with lower affinity. This is supported by the observation that inhibition of PKA activity decreases RelA DNA binding (300), an effect most probably driven by the interaction between N- and C-terminal regions of RelA, which masks the DNA binding region within the RHD of RelA. RelA S276

phosphorylation by PKA weakens this interaction allowing RelA DNA binding upon RelA nuclear translocation (181). On the other hand, DNA binding might be needed for efficient RelA phosphorylation, a notion supported by the observation that the phosphorylation of the DNA binding-deficient mutant is strongly reduced as compared to wild type RelA (285). Although we did not investigate whether S276 was hypophosphorylated in RelA DNA binding-deficient mutant, it is also plausible that the redistribution of the DNA binding-deficient mutant to the aforementioned subnuclear structures could be linked to its hypophosphorylated status.

While the RelA S276A mutant is less transcriptionally active than its wild type counterpart, this effect is not general, given that RelA S276A has comparable transcriptional activity to wild type RelA on a subset of promoters (219). One of the identified genes is the major histocompatibility (MHC) class I which is efficiently induced by the S276A mutant (219). It is noteworthy that the MHC class I gene cluster on chromosome 6 is associated with PML bodies in human primary fibroblasts (296). Moreover, PML nuclear bodies are associated with components of the transcriptional machinery such as nuclear DNA helicase II, CBP/p300, and RNA polymerase II (306, 307). This suggests that PML bodies may serve as a scaffold for factors involved in gene expression and thus direct transcriptional activity of hypophosphorylated S276 RelA to a subset of genes located on PML-associated chromatin regions. On the other hand, RelA S276A decreases the expression of IκBα, promoting RelA nuclear retention (308). Sequestration of hypophosphorylated RelA in PML nuclear bodies could therefore serve as a mechanism limiting the expression of genes efficiently transcribed by S276A in the absence of sufficient IκBα expression, thus maintaining tissue homeostasis.

V. Materials and Methods

Cell Culture

HEK293FT and COS7 cells were obtained from ATCC and grown (37 °C, 95% humidity, 5% CO_2) in Dulbecco's modified Eagle's medium (MediaTech) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin B (all Atlanta Biologicals).

Plasmid Constructs

EGFP-tagged RelA wild type (EGFP-RelA) was obtained by cloning human RelA cDNA in pEGFP-C3 vector (Clontech). EGFP-tagged RelA S276A mutant (EGFP-RelA-S276A) was generated by site-directed mutagenesis using EGFP-RelA as a template. RelA RHD S276A mutant (myc-RelA-S276A-RHD) was generated by site-directed mutagenesis using myc-RelA-RHD as a template. Catalytically inactive murine PKAcα (DN-PKA) was generated by introducing a K72M substitution by site-directed mutagenesis using pFC.PKA (Stratagene) as a template (309). Other expression constructs used in this study have been previously described including N-terminal myc-tagged RelA wild type (myc-RelA-wt) and RelA DNA binding-deficient mutant (myc-RelA-BM) (285), N-terminal myc-tagged RelA S276A mutant (myc-RelA-S276A) (219), RelA RHD wild type (myc-RelA-RHD) (301). All cloned sequences were confirmed by automated DNA sequencing using dye termination chemistry.

Antibodies

Fibrillarin, nuclear speckles and PML nuclear bodies are detected using MCA-38F3 (EnCor), S4045, (Sigma) and sc-5621 (Santa Cruz) primary antibodies, respectively. Secondary antibodies used are DyLight 646 Donkey Anti-Mouse IgG (H + L) (715-495-150, Jackson

ImmunoResearch) and DyLight 646 Donkey Anti-Rabbit IgG (H + L) (711-495-152, Jackson ImmunoResearch).

Transient transfection

HEK293FT cells were seeded on collagenized cover slips in a 12-well plate ($2.5x10^5$ cells per well) and transfected at 90% confluency using calcium phosphate method. Cells were exposed to 1.6 μg total DNA and 0.125 M $CaCl_2$, 1X HeBS. COS-7 cells were seeded on collagenized cover slips in a 12-well plate ($1x10^5$ cells per well) and transfected at 90% confluency. Cells were exposed to 0.3 μg of total DNA and 2 μl of Lipofectamine reagent, according to manufacturer's instruction (Invitrogen) in Dulbecco's modified Eagle's medium for 5h. After addition of fetal bovine serum to a final concentration of 10%, cells were incubated (one day, 37 °C) and used for immunostaining. The amounts of EGFP-RelA indicator protein and corresponding effector RelA protein used for transfections were 20ng and 200ng, respectively in HEK293FT cells and 50ng and 250ng, respectively in COS7 cells.

Immunostaining

One day post-transfected HEK293FT or COS7 cells were washed in phosphate-buffers saline (PBS), fixed (4% paraformaldehyde in PBS, 10 min, RT), permeabilized (0.5 % Triton X-100 in PBS, 5 min, RT) and subsequently blocked (1% donkey serum, 0.05 % Tween 20 in PBS, 20 min, RT). Cells were incubated with specific primary antibodies (1% serum, 0.05 % Tween 20 in PBS, 1 h, RT), washed and incubated with fluorochrome-conjugated secondary antibodies (1% serum, 0.05 % Tween 20 in PBS, 1 h, RT). Nuclear DNA was stained with TO-PRO-3 (1 mM; Invitrogen) when indicated and cover slips were mounted with SlowFade Gold antifade reagent (Invitrogen). Otherwise, cover slips were mounted with SlowFade Gold antifade reagent containing DAPI

(Invitrogen). Subcellular distribution of EGFP-tagged RelA, nucleolus, nuclear speckles and PML nuclear bodies was analyzed using either Nikon Eclipse TE2000 microscope equipped with a 40x objective or a Leica confocal laser-scanning microscope equipped with 60x objective. Pictures were acquired with a charge-coupled device and images were processed with IPLab software (Scanalytics, Vers. 3.9.3).

IV. Author contribution

N. Pejanovic and J. Anrather planed the experiments and N. Pejanovic performed the experiments described in this chapter.

CHAPTER IV
REGULATION OF NF-kB ACTIVITY BY PHOSPHORYLATION *

*Adapted from Seldon MP‡, Silva G‡, **Pejanovic N**, Larsen R, Gregoire IP, Filipe J, Anrather J, and Soares MP. 2007. *Heme oxygenase-1 inhibits the expression of adhesion molecules associated with endothelial cell activation via inhibition of NF-κB RelA phosphorylation at serine 276. J Immunol*

‡ Seldon MP and Silva G. contributed equally to this work

I. Summary

Heme oxygenase-1 (HO-1; encoded by the *Hmox1* gene) catalyzes the degradation of free heme into biliverdin (BV), via a reaction that releases iron (Fe) and carbon monoxide (CO). We report that HO-1 down-regulates the proinflammatory phenotype associated with endothelial cell (EC) activation by reducing intracellular nonprotein-bound Fe (labile Fe). EC isolated from *Hmox1*$^{-/-}$ mice have higher levels of intracellular labile Fe and reactive oxygen species (ROS) as compared with EC isolated from *Hmox1*$^{+/+}$ mice. Basal and tumor necrosis factor (TNF)-induced expression of vascular adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin were increased in *Hmox1*$^{-/-}$ *vs Hmox1*$^{+/+}$ EC, an effect reversed by Fe chelation using deferoxamine mesylate (DFO). Fe chelation inhibits TNF-driven transcription of *Vcam-1*, *Icam-1*, and *E-selectin*, as assessed using luciferase reporter assays. This effect is associated with inhibition of the transcription factor (TF) nuclear factor kappa B (NF-κB) via a mechanism that is not associated with the inhibition of inhibitor of κB α (IκBα) phosphorylation/degradation or NF-κB (i.e. RelA) nuclear translocation, although it affects very modestly NF-κB binding to DNA κB consensus sequences in the *Vcam-1* and *E-selectin* promoters. HO-1 inhibits NF-κB (i.e. RelA) phosphorylation at serine (S) 276, a phospho-acceptor that is critical to sustain TNF-driven NF-κB activity in EC. This effect was mimicked by Fe chelation as well as by antioxidant *N*-acetylcysteine (NAC). In conclusion, we demonstrate a novel mechanism via which HO-1 down-modulates the proinflammatory phenotype of activated EC, i.e. the inhibition of RelA phosphorylation at S276.

II. Background

Under homeostasis, EC regulate blood flow and orchestrate leukocyte trafficking throughout tissues, via expression of genes that regulate vasoconstriction, thrombosis and inhibit leukocyte adhesion (310, 311). Under inflammatory conditions however, EC undergo profound phenotypic modifications, becoming highly vasoconstrictive, pro-thrombotic and pro-adhesive, a phenomenon referred to as EC activation (310). These phenotypic modifications rely on the down-regulation of the expression of vasodilatory, anti-thrombotic and anti-adhesive genes as well as on the induced expression of several immediate early-responsive vasoconstrictive, pro-thrombotic and pro-adhesive genes (311). Expression of these proinflammatory genes occurs through a mechanism that requires the activation of the NF-κB (312, 313).

The NF-κB family of TF is composed of five members sharing a consensus Rel homology domain (RHD), i.e. p50, p52, cRel, RelB, and RelA (314), the latter of which is probably the predominant active form in EC (140). NF-κB proteins form homodimers and heterodimers that can bind κB DNA motifs in the promoter regions of "NF-κB-dependent genes" (315). In quiescent EC, NF-κB dimers are maintained mostly in the cytoplasm by virtue of their interaction with IκB molecules that occlude the nuclear localization signals (NLS) (88, 316) in their RHD. However, when exposed to proinflammatory agonists such as TNF, IκBs are phosphorylated, polyubiquitinated, and degraded via the 26S proteosome pathway (317) so that nuclear localization signal exposure on NF-κB dimers allows for nuclear translocation and binding to DNA κB motifs (88).

There are additional mechanisms that modulate NF-κB

transcriptional activity (86). These include, but are not restricted to, phosphorylation of S205 (219), S276 (185, 285, 300), S281 (219), and S311 (186) in the N-terminal domain of RelA. Modulation of these phospho-acceptors is under the control of several kinases, including protein kinase A (PKA) (300), mitogen- and stress-activated kinase-1 (MSK1) (185), and protein kinase C ζ (PKCζ) (186, 285). The phosphorylation status of RelA controls not only its transcriptional activity but also specificity for different subsets of target genes (219). How these phospho-acceptors modulate RelA activity is not clear but presumably involves the interaction of RelA with co-activators such as the cyclic AMP-responsive element binding protein (CREB)-binding protein (CBP)/p300 (300, 318).

Although essential to elicit inflammatory responses, the expression of proinflammatory genes associated with EC activation must be tightly regulated so as to prevent unfettered inflammation. One of the mechanisms via which this occurs relies on the expression of "protective genes" (319). These have a dual role in that they regulate not only NF-κB activity but in addition protect EC from undergoing apoptosis (319). We have previously demonstrated that the stress responsive enzyme HO-1 (encoded by the *Hmox1* gene) acts in such a manner (320, 321).

When exposed to proinflammatory agonists, EC up-regulate the expression of HO-1 via a mechanism that probably requires the relief of its constitutive transcriptional repression by the broad-complex tramtrack and bric a brac (BTB) and cap'and'collar (CNC) homologue 1 (BACH-1) (322, 323). Once BACH-1 is released from the *Hmox1* promoter, binding of the transcription activator NF-E2-related factor-2 (NRF-2) occurs, leading to *Hmox1* transcription (323).

Under inflammatory conditions, HO-1 becomes the rate-limiting enzyme in the catabolism of heme, a reaction that releases Fe from the inner core of the protoporphyrin ring of heme and generates BV as well

as the gas CO (52). The free Fe generated in this manner up-regulates the expression of heavy chain ferritin (FtH) (54), which forms a multimeric complex with the light chain ferritin and nucleates free Fe, impairing it from partaking in the generation of ROS via the Fenton reaction (324), a highly deleterious effect (325-327). Free Fe also induces the expression/activity of an ATPase Fe efflux pump that decreases cellular Fe content (328).

BV, another end product of heme catabolism by HO-1 (52), is converted into the antioxidant bilirubin (BR) (53) by biliverdin reductase (329). Although BR can inhibit NF-κB activation in EC (20), the relatively low level of biliverdin reductase expressed in EC makes it unlikely that this antioxidant would act as an autocrine manner to regulate NF-κB activity. Therefore, we reasoned that HO-1 might control NF-κB activation in EC via its ability to down-modulate the levels of intracellular labile Fe. We provide evidence that the reduction of EC labile Fe content associated with HO-1 expression inhibits specifically the phosphorylation of RelA at S276 and probably at S205, thus reducing NF-κB transcriptional activity. This mechanism underlies the inhibition of proinflammatory gene expression associated with the expression of HO-1 in EC and should contribute in a critical manner to the resolution of inflammatory reactions such as afforded by HO-1 expression.

III. Results

1. Reduction of EC labile Fe associated with HO-1 expression down-modulates VCAM-1 expression

HO-1 deficient mice (70) and humans (71) have deregulated Fe metabolism and widespread EC activation/injury, suggesting that HO-1 expression is essential in the regulation of Fe homeostasis (70, 330) and

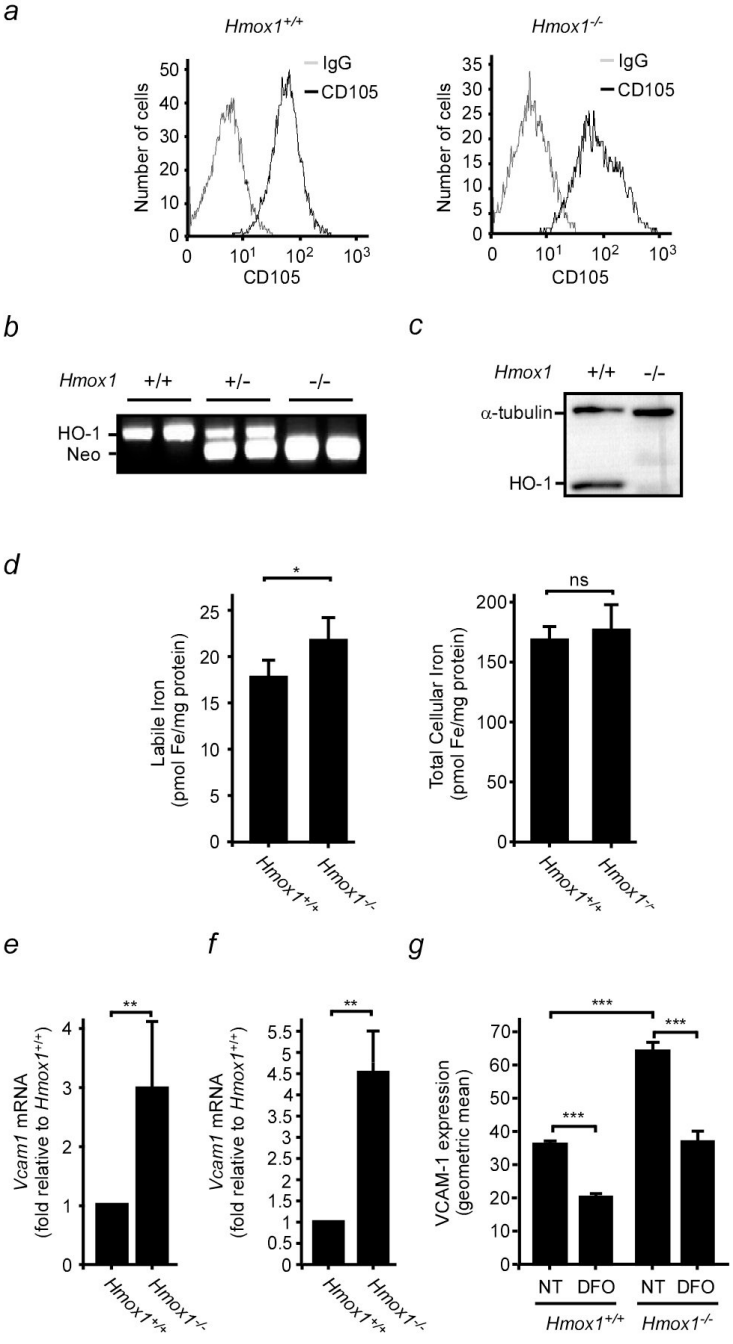

a
Hmox1+/+
IgG
CD105
Number of cells
CD105
Hmox1-/-
b
Hmox1
+/+
+/-
-/-
HO-1
Neo
c
α-tubulin
HO-1
d
Labile Iron
(pmol Fe/mg protein)
Total Cellular Iron
ns
e
Vcam1 mRNA
(fold relative to Hmox1+/+)
f
g
VCAM-1 expression
(geometric mean)
NT
DFO

Figure 4.1. HO-1 regulates VCAM-1 expression via modulation of EC labile Fe content. a) Endoglin/CD105 was detected by flow-cytometry in MEC from Hmox1+/+ or Hmox1-/- BALB/c mice. Grey histograms represent control IgG staining and black histograms anti-CD105 staining. **b)** Hmox1+/+, Hmox1+/- and Hmox1-/- genotypes were verified by PCR. **c)** HO-1 protein was detected by western blot using whole cell extracts from Hmox1+/+ and Hmox1-/- MEC. **d)** Labile and total Fe were quantified in Hmox1+/+ and Hmox1-/- MEC. Results are shown as mean ± standard deviation from 6 independent samples in one out of two independent experiments. **e)** Vcam-1 mRNA expression in the liver of Hmox1+/+ and Hmox1-/- mice, quantified by qRT-PCR. Values are normalized to Hprt mRNA and shown as mean fold increase ± standard deviation, relative to Hmox1+/+ (n=3 pair matched animals for each genotype). Statistical significance was assessed using one-sample z-test. **f**) Vcam-1 mRNA expression in EC from Hmox1+/+ or Hmox1-/- mice, quantified by qRT-PCR. Values are normalized and shown as in (e) (n=3 independent wells in one out of two experiments). **g)** Surface VCAM-1 expression in EC from Hmox1+/+ or Hmox1-/- mice treated or not with DFO (250μM, 16h) was detected by flow-cytometry. Results shown are geometric mean fluorescence ± standard deviation from triplicate samples in one out of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ns = not significant (p>0.05).

in preventing unfettered EC activation. Because a causal link between deregulated Fe metabolism and unfettered EC activation has not been established, we asked whether deregulated Fe metabolism would promote EC activation. Primary EC were isolated from *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ mice and their phenotype was confirmed by flow cytometry, i.e. expression of endoglin/CD105 (see Figure 4.1.a). Genotype was confirmed by PCR (see Figure 4.1.b) and Western blotting (see Figure 4.1.c). Labile Fe content increased by 23% in *Hmox1*$^{-/-}$ *vs Hmox1*$^{+/+}$ EC (see Figure 4.1.d), while non-labile Fe content was similar whether or not HO-1 was expressed (see Figure 4.1.d), suggesting that HO-1 regulates specifically the labile Fe content of EC.

We then asked whether increased labile Fe content resulting from *Hmox1* deletion would modulate the expression of adhesion

molecules in EC. Expression of *Vcam-1* mRNA, an adhesion molecule associated with EC activation (310, 311), was increased by 3- to 4-fold in the liver of *Hmox1*$^{-/-}$ vs Hmox1$^{+/+}$ mice as quantified by quantitative real-time PCR (see Figure 4.1.e). To ascertain that HO-1 expression in EC accounted for this effect, *Vcam-1* mRNA expression was quantified specifically in EC isolated from *Hmox1*$^{-/-}$ or *Hmox1*$^{+/+}$ mice. The basal level of *Vcam-1* mRNA expression increased by 4- to 5-fold in EC from *Hmox1*$^{-/-}$ vs Hmox1$^{+/+}$ mice (see Figure 4.1.f). Expression of VCAM-1 protein was also significantly increased in *Hmox1*$^{-/-}$ vs Hmox1$^{+/+}$ EC (see Figure 4.1.g). This effect was probably due to higher levels of labile Fe in *Hmox1*$^{-/-}$ EC (see Figure 4.1.d), as Fe chelation by DFO reduced VCAM-1 expression in *Hmox1*$^{-/-}$ EC to levels similar to those of *Hmox1*$^{+/+}$ EC (see Figure 4.1.g). Taken together, these observations suggest that HO-1 expression in EC might be a physiologic regulator of basal VCAM-1 expression, an effect that probably relies on the control of labile Fe.

2. Down-regulation of labile Fe associated with the expression of HO-1 inhibits TNF-driven up-regulation of adhesion molecules in EC

We have previously shown that when overexpressed in EC, HO-1 inhibits the expression of adhesion molecules associated with EC activation, including VCAM-1, E-selectin, and ICAM-1 (321). We now asked to what extent this effect is associated with decreased labile Fe content (see Figure 4.1.d). Transient HO-1 overexpression in EC reduced labile Fe content by 17% as compared with control EC (see Figure 4.2.a). A similar effect was observed when nontransfected EC were treated with the Fe chelator DFO, i.e., an 18% reduction in labile Fe content as compared with untreated EC (see Figure 4.2.a). TNF, a well-established proinflammatory agonist, induced a transient increase of

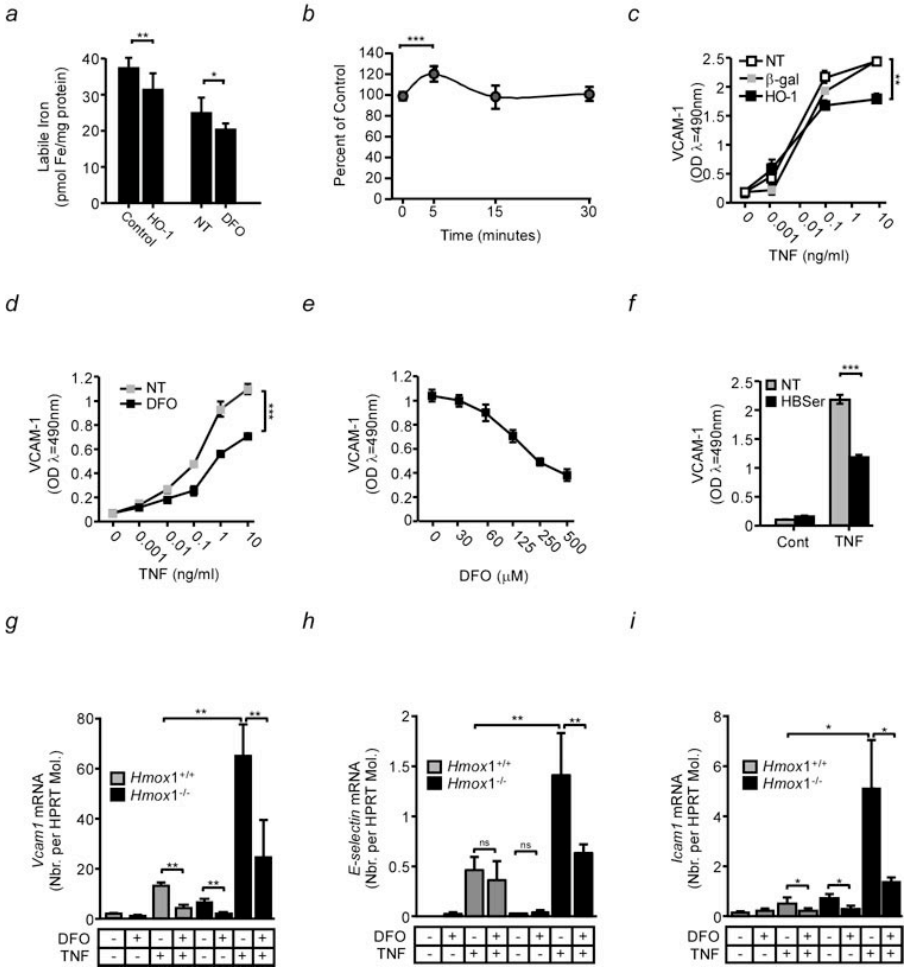


Figure 4.2. HO-1 inhibits TNF-driven expression of VCAM-1, E-selectin and ICAM-1 via reduction of labile Fe content. a) Labile Fe was quantified in BAEC transiently transfected with control or HO-1 expression vectors, as well as in non-transfected BAEC treated or not (NT) with DFO (250 μM, 16h). Mean values ± standard deviation (9 independent samples). **b)** Labile Fe was quantified in confluent BAEC not-treated (0) or treated with TNF (50 ng/ml). Mean values ± standard deviation from triplicate samples (one out of three representative experiments). **c)** Non-transduced, β-galactosidase and HO-1 recombinant adenoviruses transduced HUVEC (24h) were stimulated with TNF (6h). VCAM-1 was detected by cellular ELISA. **d)** HUVEC treated with DFO (250 μM, 16h) and stimulated with increasing

concentrations of TNF (6h). VCAM-1 was detected by cellular ELISA. **e)** HUVEC were treated with increasing concentrations of DFO (1h) and stimulated with TNF (10ng/ml). VCAM-1 expression was assessed as in (c) and (d). Results shown in (c), (d) and (e) are mean values ± standard deviation from triplicate samples in one out of three independent experiments. **f)** HUVEC were not-treated (NT) or treated with the Fe chelator HBSer (5 mM, 16h) and not further treated (Cont) or exposed to TNF (0.1 ng/ml, 8h). Mean values ± standard deviations (triplicate samples in one out of four independent experiments). **g, h and i**) Confluent MEC from Hmox1-/- or Hmox1+/+ mice were treated with DFO (250 µM, 16h) and TNF−α (50 ng/ml; 3h) when indicated (+). Vcam-1 (**g**), E-selectin (**h**) and Icam-1 (**i**) mRNA were quantified by qRTPCR. Values are normalized to Hprt mRNA and shown as mean number of molecules ± standard deviation (n=3 independent wells in one representative experiment). *p<0.05, **p<0.01, ***p<0.001, ns = not significant (p>0.05).

12–20% in EC labile Fe content (see Figure 4.2.b), an effect in keeping with that reported in other cell types (331). HO-1 overexpression (see Figure 4.2.c) or Fe chelation by DFO (see Figure 4.2.d) reduced by 30–40% the ability of TNF to up-regulate VCAM-1 expression in EC. The inhibitory effect of Fe chelation was dose-dependent in that higher DFO concentrations enhanced its effect (see Figure 4.2.e). To exclude the possibility that DFO would function independently of its Fe-chelating activity, experiments were performed using HBSer, a "milder" Fe chelator (332). HBSer mimicked the effect of DFO, but only when TNF concentrations were <1 ng/ml, i.e., a 45% reduction in VCAM-1 expression, as compared with control EC (see Figure 4.2.f). Similar results were obtained using SIH, a cell-permeable Fe chelator (333) (data not shown). These data suggest that despite their relative differences in efficiency, it is the Fe chelation activity of these compounds, i.e., DFO, HbSer and SIH, which affords their effects.

To ascertain whether, when expressed under physiological conditions, HO-1 controls the induction of VCAM-1 expression in response to TNF, *Vcam-1* mRNA expression was quantified by

quantitative real-time PCR after the exposure of *Hmox1*$^{-/-}$ or *Hmox1*$^{+/+}$ EC to TNF. Accumulation of *Vcam*-1 mRNA was ~5-fold higher in *Hmox1*$^{-/-}$ vs *Hmox1*$^{+/+}$ EC (see Figure 4.2.g). Higher *Vcam*-1 mRNA expression in *Hmox1*$^{-/-}$ EC was ablated by DFO, suggesting that it is the higher labile Fe content in *Hmox1*$^{-/-}$ EC that accounts for this effect (see Figure 4.2.g).

We then asked whether, when expressed under physiological conditions, HO-1 would control the expression of other TNF-responsive adhesion molecules in EC. Up-regulation of *E-selectin* (see Figure 4.2.h) and *Icam-1* (see Figure 4.2.i) mRNA in response to TNF was increased by ~3- and ~11-fold in *Hmox1*$^{-/-}$ vs *Hmox1*$^{+/+}$ EC, respectively. Higher *E-selectin* (see Figure 4.2.h) and *Icam-1* (see Figure 4.2.i) mRNA expression in *Hmox1*$^{-/-}$ EC was ablated by DFO, suggesting again that it is the higher labile Fe of *Hmox1*$^{-/-}$ EC that accounts for this effect. Taken together, these data suggest that, when expressed under physiological conditions, HO-1 reduces the expression of adhesion molecules associated with EC activation, most probably via down-modulation of labile Fe.

3. Fe chelation inhibits the transcription of genes encoding adhesion molecules in EC

We tested whether down-modulation of labile Fe inhibits *Vcam-1*, *E-selectin*, or *Icam-1* transcription. EC were transiently transfected with a *VCAM-1*-luc reporter and treated or not treated with DFO. Induction of *VCAM-1* reporter activity by TNF was reduced by 77–100% in EC treated with DFO vs control EC (see Figure 4.3.a). This effect was dose dependent in that increasing DFO concentrations (60–250 μM) enhanced its inhibitory effect (see Figure 4.3.a). The inhibitory effect of DFO was observed across a wide range of TNF concentrations (0.16–

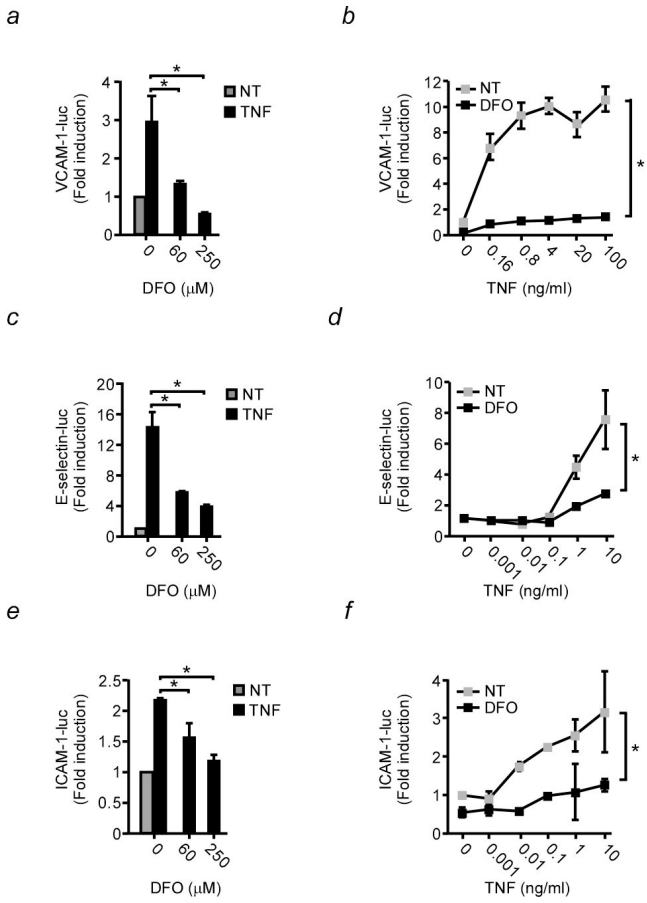


Figure 4.3. Fe chelation inhibits TNF-driven Vcam-1, E-selectin and Icam-1 transcription. a) and **b)** BAEC were transiently transfected with a VCAM-1 luciferase reporter (46) and treated with **a)** increasing concentrations of DFO (16h) and TNF (10 ng/ml, 6h) or **b)** DFO (250 μM, 16h) and increasing concentrations of TNF (6h). **c)** and **d)** BAEC were transiently transfected with an E-selectin luciferase reporter (46) and **c)** treated as in (a) or **d)** treated as in (b). **e)** and **f)** BAEC were transiently transfected with an ICAM-1 luciferase (46) reporter and **e)** treated as in (a) or **f)** treated as in (b). Results shown are mean luciferase normalized to β-galactosidase units ± standard deviation, from triplicate samples in one out of three independent experiments. (*$p<0.05$)

100 ng/ml) (see Figure 4.3.b). Finally, this effect was not associated with detectable cytotoxicity, as assessed by flow cytometry (data not shown).

DFO also reduced TNF-driven *E-selectin* reporter expression by 65–80% as compared with control EC (see Figure 4.3.c). Higher DFO concentrations (60–250 μM) afforded higher inhibition (see Figure 4.3.c), an effect observed over a broad range of TNF concentrations (0.001–10 ng/ml) (see Figure 4.3.d). Similarly, DFO reduced TNF-driven *Icam-1* reporter expression by 20–45%, again in a dose dependent manner (see Figure 4.3.e) and over a broad range of TNF concentrations (0.001–10 ng/ml) (see Figure 4.3.f). Taken together these observations suggest that down modulation of Fe content impairs TNF from inducing *Vcam-1*, *E-selectin*, and *Icam-1* transcription in EC.

4. Reduction of EC labile Fe content inhibits NF-κB activity

Induction of *Vcam-1*, *E-selectin*, and *Icam*-1 transcription by TNF is strictly dependent on activation of the transcription factor NF-κB in EC (301, 312, 313, 320). Therefore, we reasoned that the reduction of labile Fe might inhibit NF-κB activation (see Figure 4.3.). EC were transiently transfected with a synthetic NF-κB-luc reporter derived from the porcine *E-selectin* promoter (284) plus or minus an HO-1 expression vector. TNF-driven NF-κB reporter activity was reduced by 42–57% in EC transfected with HO-1 as compared with control EC (see Figure 4.4.a). The inhibitory effect of HO-1 was dose dependent; i.e., higher HO-1 expression resulted in lower NF-κB reporter activity (see Figure 4.4.a). This effect was observed over a broad range of TNF concentrations (1–10 ng/ml) (see Figure 4.4.b). Given that HO-1 overexpression reduces the labile Fe content of EC (see Figure 4.2.a), we asked whether Fe chelation would mimic the inhibitory effect of HO-1. DFO inhibited TNF-driven NF-κB reporter activity by 63–78% as compared with control EC

not treated with DFO (see Figure 4.4.c). This effect was dose dependent; i.e., higher DFO concentrations resulting in lower NF-κB reporter activity (see Figure 4.4.c), and this was observed over a broad range of TNF concentrations (1–10 ng/ml) (see Figure 4.4.d). These data suggest that HO-1 inhibits NF-κB activation via a mechanism probably involving the reduction of labile Fe content in EC.

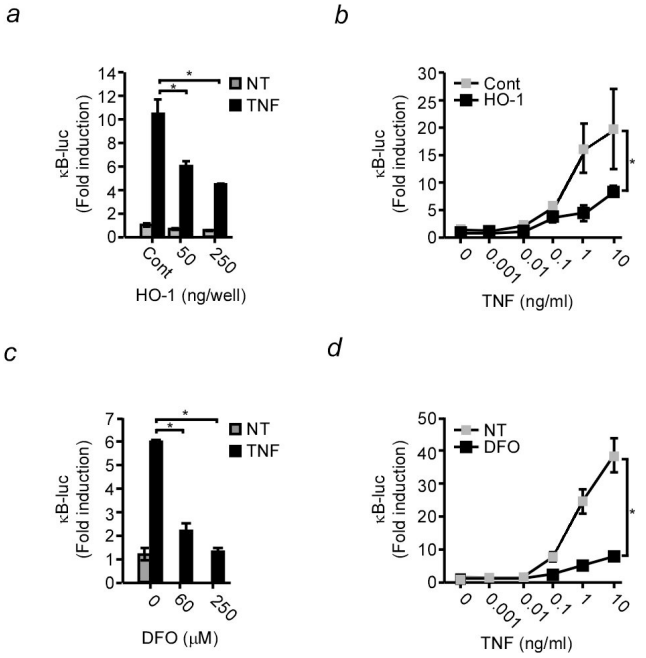


Figure 4.4. HO-1 over-expression and Fe chelation inhibit NF-κB activation in EC. **a)** BAEC were transiently co-transfected with a NF-κB luciferase reporter derived from the porcine E-selectin promoter (43) (κB-luc) plus pcDNA3-HO-1 (3000 ng DNA/3x105 cells) and stimulated with TNF (10 ng/ml, 6 h) or not (NT). **b)** BAEC were transiently transfected as in (a) and stimulated with increasing concentrations of TNF. **c)** BAEC were transiently transfected with a NF-κB luciferase reporter (κB-luc), treated with DFO (16h) and stimulated with TNF (10 ng/ml, 6 h) or not (NT). **d)** BAEC were transiently transfected as in (c), treated with DFO (250 μM, 16h) and stimulated with TNF (6h). Results shown are mean luciferase normalized to β-galactosidase units ± standard deviation from triplicate samples in one out of three independent experiments. *p<0.05.

5. Reduction of labile Fe in EC does not affect IκBα phosphorylation/degradation or RelA nuclear translocation

When exposed to DFO under the same experimental conditions shown to inhibit NF-κB activation (see Figures 4.4.c and 4.4.d), TNF-driven IκBα phosphorylation and/or IκBα degradation was not affected as compared with control EC (see Figure 4.5.a). DFO also did not alter de novo IκBα synthesis after TNF stimulation as compared with control EC (see Figure 4.5.a). In addition, DFO failed to modulate the levels of RelA protein expression or RelA phosphorylation at the C-terminal region, i.e., S536, as compared with control EC (see Figure 4.5.a). RelA nuclear translocation in response to TNF was also not affected by DFO as compared with control EC (see Figure 4.5.b). Total nuclear RelA was also not significantly modulated by DFO as assessed by Western blot analysis (see Figure 4.5.c) (334).

6. Fe chelation interferes modestly with NF-κB binding to specific κB DNA binding motifs in the promoter region of Vcam-1, E-selectin, or Icam-1

Given that Fe chelation failed to modulate IκBα phosphorylation/degradation (see Figure 4.5.a) and/or RelA nuclear translocation (see Figures 4.5.b and 4.5.c), we reasoned that inhibition of NF-κB activity (see Figure 4.4.) might occur "downstream" of nuclear translocation. Binding of NF-κB to κB DNA binding motifs derived from the *Vcam-1*, *E-selectin*, and *Icam-1* promoters was compared by EMSA, using nuclear extracts from EC pre-exposed or not pre-exposed to DFO (16 h) and stimulated or not stimulated thereafter with TNF (60 min). DFO failed to modulate NF-κB binding to a "standard" κB binding motif derived from the Ig promoter (Ig-κB) as compared with control EC (see Figure 4.5.d). In a similar manner, DFO failed to modulate the very

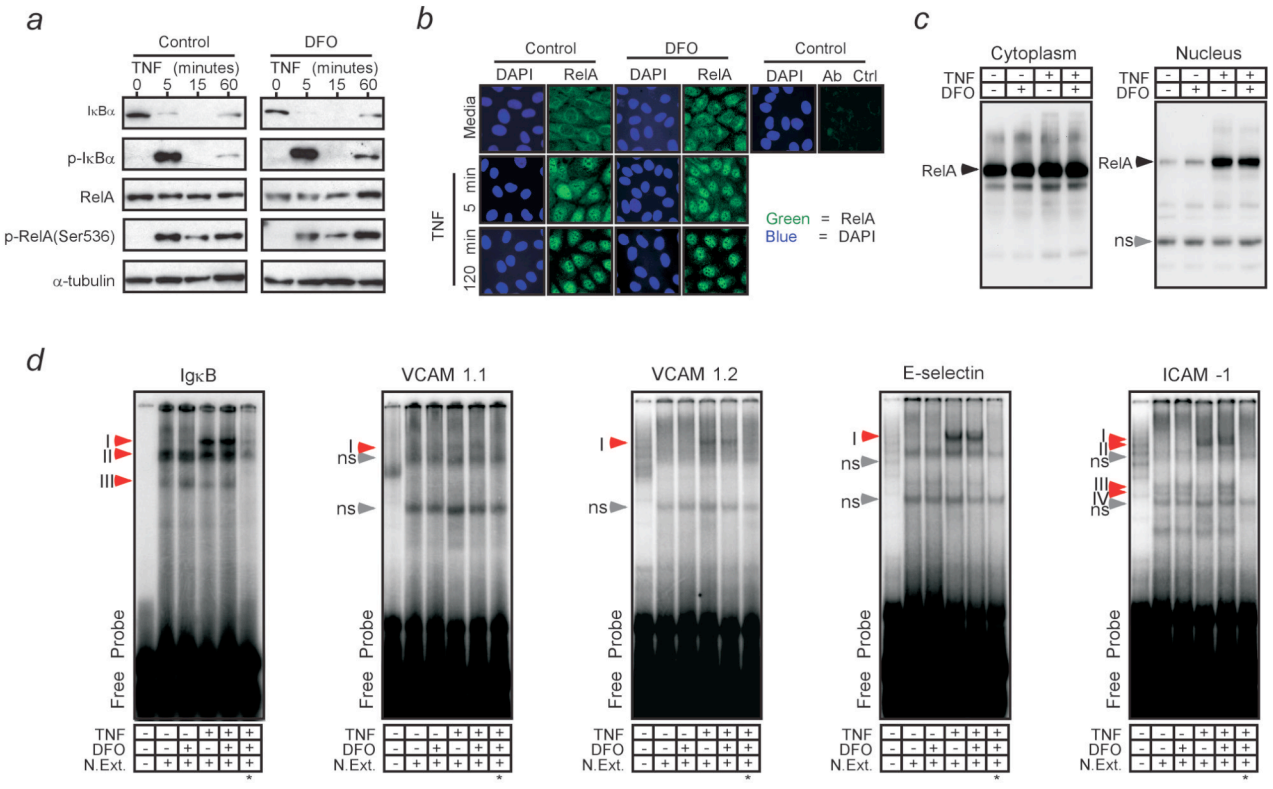

a
Control
DFO
TNF (minutes)
0 5 15 60
IκBα
p-IκBα
RelA
p-RelA(Ser536)
α-tubulin
b
Control
DFO
Control
DAPI RelA DAPI RelA DAPI Ab Ctrl
Media
TNF 5 min
TNF 120 min
Green = RelA
Blue = DAPI
c
Cytoplasm
Nucleus
TNF
DFO
RelA
ns
d
IgκB
VCAM 1.1
VCAM 1.2
E-selectin
ICAM -1
I
II
III
IV
ns
Free Probe
TNF
DFO
N.Ext.

Figure 4.5. Fe chelation does not affect IκBα phosphorylation/degradation or NF-κB nuclear translocation while inhibiting moderately NF-κB binding to specific κB DNA binding motifs in the *Vcam-1*, *E-selectin* and *Icam-1* promoters. ***a)*** Confluent BAEC either not treated (control) or pre-treated with DFO (250 μM, 16h) were stimulated with TNF (10 ng/ml). IκBα, phosphorylated IκBα (p-IκBα), RelA and Ser536 phosphorylated RelA (p-RelA-Ser536) were detected by western blot. Immunoblots are representative of three independent experiments. ***b)*** BAEC treated as in (a), RelA (green) and DNA (blue) were immunostained with anti-RelA antibody and DAPI, respectively. Images (400x) were acquired by fluorescence microscopy and are representative of four fields from one out of three representative experiments. ***c***) BAEC were treated as in (a). RelA was monitored in cytoplasm and nucleus by western blot. ***d)*** BAEC were treated as in (a). EMSA were performed using nuclear extracts (N.Ext) plus radiolabeled oligonucleotides corresponding to the κB binding sites from the human Ig locus (Ig-κB), *Vcam1* (-167bp; VCAM-1.1 and -152bp; VCAM-1.2), *E-selectin* (-99bp) and *Icam1* (- 1390bp) promoters. Specific (red arrows; I-IV) and non-specific (black arrows, ns) complexes are shown. Binding specificity was assessed using for each probe using 20 fold molar excess of cold probe (*).

modest binding of NF-κB to the –167-bp *Vcam-1*κB binding motif (VCAM1.1). There was a slight but reproducible inhibition of NF-κB binding to the –152-bp *Vcam-1*κB binding motif (VCAM1.2) in EC treated with DFO, as compared with control EC (see Figure 4.5.d). DFO reduced very slightly NF-κB binding to the –99bp *E-selectin* κB binding motif while not affecting NF-κB binding to the –1390-bp *Icam-1*κB DNA binding motif (see Figure 4.5.d) as compared with control EC. In all cases, the characteristic pattern of NF-κB/DNA binding was observed, namely the appearance of "specific bands" upon TNF stimulation that were competed out using 20-fold excess unlabeled κB probe, corresponding to the probe used in each EMSA, respectively (see Figure 4.5.d).

7. Fe chelation inhibits RelA transcriptional activity

Because the inhibition of NF-κB activation by HO-1 or Fe chelation (see Figure 4.4.) was not associated with the inhibition of NF-κB nuclear translocation (see Figures 4.5.b and 4.5.c), and only to a modest extent with DNA binding (see Figure 4.5.d), we tested whether the inhibitory effect of HO-1 acted directly on RelA, presumably the main NF-κB transactivator in EC (140). EC from *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ mice were transiently transfected with increasing amounts of RelA plus an NF-κB luciferase reporter derived from the porcine *E-selectin* promoter (284). RelA activity was 2–3-fold higher in EC from *Hmox1*$^{-/-}$ mice as compared with EC from *Hmox1*$^{+/+}$ mice (see Figure 4.6.a). This suggests that, when expressed under physiologic conditions, HO-1 inhibits RelA activity.

We then asked whether Fe chelation would inhibit RelA activity. Expression of the same NF-κB luciferase reporter was increased by ~40-fold when EC were co-transfected with RelA, RelA plus p50, or RelA plus cRel expression vectors as compared with control EC that did not overexpress these NF-κB family members (see Figure 4.6.b). Fe chelation by DFO inhibited RelA-, RelA plus p50-, and RelA plus cRel-driven reporter transactivation by ~45–57% as compared with control EC (see Figure 4.6.b). Fe chelation did not alter RelA expression as assessed by Western blot analysis using an anti-Myc-tagged Ab recognizing specifically overexpressed RelA (see Figure 4.6.c). Inhibition of RelA activity was also observed in HEK293 cells transiently co-transfected with RelA and the same NF-κB luciferase reporter (see Figure 4.6.d), illustrating that this effect is not restricted to EC.

We then asked whether Fe chelation would inhibit endogenous *Vcam-1*, *E-selectin*, and *Icam-1* transcription when driven specifically by RelA. For this purpose, *RelA*$^{-/-}$ MEF were transduced or not transduced

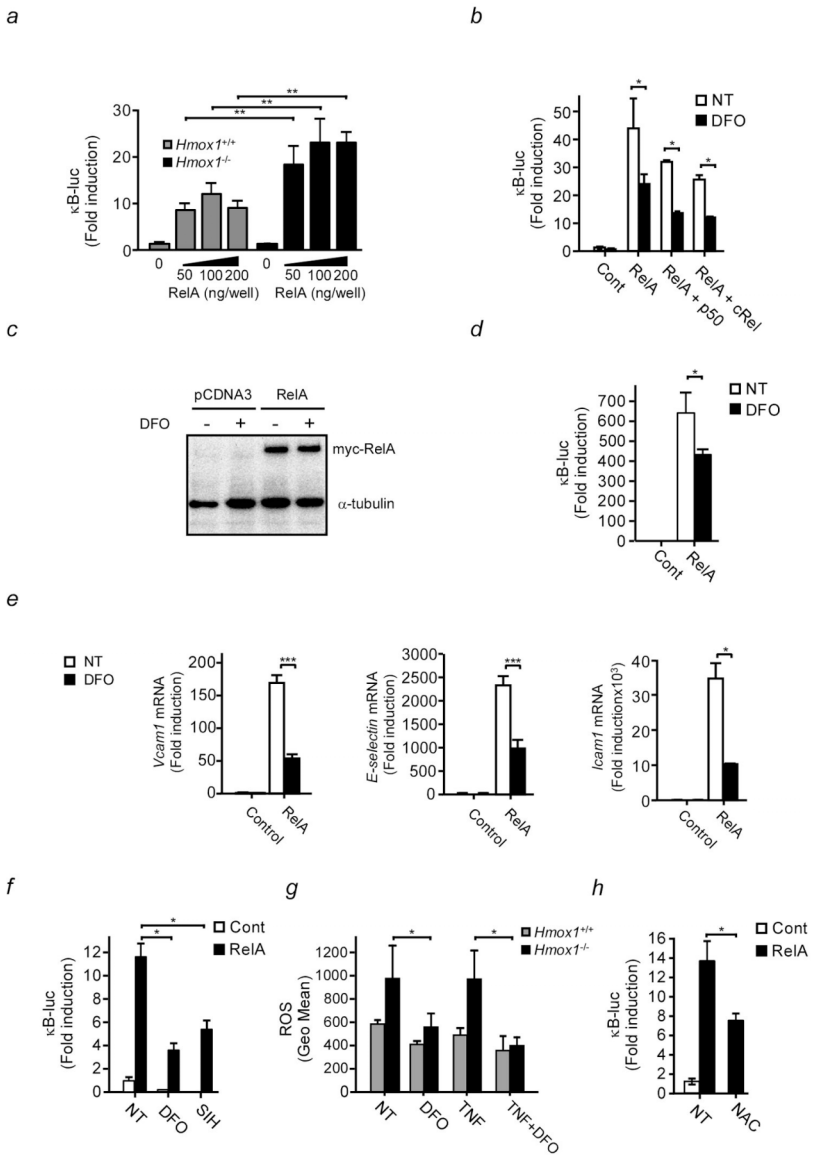


Figure 4.6. Fe chelation inhibits RelA transcriptional activity. a) Hmox1+/+ and Hmox1-/- EC transiently co-transfected with RelA and a NF-κB luciferase reporter derived from the porcine E-selectin promoter (κB-luc)(43). Mean fold induction of luciferase/β-galactosidase (compared to EC not transfected with RelA) ± standard deviation (triplicates in one out of two independent experiments). **b)** BAEC transiently co-transfected with the same NF-κB luciferase reporter as in (a) plus control pcDNA3

(Cont), RelA, RelA plus p50 or RelA plus cRel. Cells were either not treated (NT) or treated with DFO (250 μM, 16h). Mean fold luciferase/β-galactosidase induction versus control (cont) EC ± standard deviation (triplicate samples in one out of three independent experiments). **c)** Over-expressed RelA was detected by western blot using an antibody recognizing the c-myc tag in the N-terminus of RelA. **d)** HEK-293 cells were transiently co-transfected and treated as in (b). **e)** RelA-/- MEF were transduced with a control or a RelA recombinant retrovirus and treated (NT) as in (a). mRNA was quantified by qRT-PCR. Mean fold luciferase/β-galactosidase induction versus control (cont) EC ± standard deviation (triplicate samples in one experiment). **f)** BAEC were transiently co-transfected and treated with DFO (250 μM, 16h) or SIH (100 μM, 16h). Mean fold luciferase/β-galactosidase induction versus control untreated (NT) EC ± standard deviation (triplicate samples in one out of three independent experiments). **g**) Hmox1+/+ or Hmox1-/- EC were pre-treated or not with DFO (250 μM; 12-16h) and exposed or not to TNF (50 ng/ml; 1h). ROS were detected by flowcytometry using CM-H2DCFDA. Geo means ± standard deviation (one triplicate sample in one out of four independent experiments). **h)** BAEC were transiently transfected as in (f) and when indicated treated with NAC (25 μM, 16h). Mean fold luciferase/β-galactosidase induction versus control untreated (NT) EC ± standard deviation (triplicate samples in one out of three independent experiments). *p<0.05, **p<0.01, ***p<0.001.

with a recombinant RelA retrovirus, allowing for the assessment of RelA-driven *Vcam-1*, *E-selectin*, and *Icam-1* mRNA transcription by quantitative real-time PCR. *RelA*$^{-/-}$ MEF reconstituted with RelA expressed *Vcam-1*, *E-selectin*, and *Icam-1* mRNA transcripts that were ~150-, ~2500-, and ~30.000-fold more abundant to those detected in *RelA*$^{-/-}$ MEF, respectively (see Figure 4.6.e). DFO inhibited by 69, 58, and 70% *Vcam-1*, *E-selectin*, and *Icam-1* mRNA expression driven by RelA, respectively (see Figure 4.6.e). This observation provides direct evidence that Fe chelation inhibits RelA-mediated transcription of endogenous *Vcam-1*, *E-selectin*, and *Icam-1*.

As for DFO, SIH, a cell-permeable Fe chelator (333), also inhibited RelA activity as assessed in EC transiently co-transfected with

RelA plus NF-κB luciferase reporter (see Figure 4.6.f). This suggests that the inhibitory effect of these molecules is due to their Fe-chelating activity. We reasoned that the reduction of EC labile Fe content might down-regulate RelA activity by disabling labile Fe from promoting the generation of ROS through the Fenton reaction. If this is the case, then HO-1 expression should inhibit ROS accumulation in EC. Intracellular ROS content was ~2-fold higher in EC isolated from *Hmox1*$^{-/-}$ vs *Hmox1*$^{+/+}$ mice (see Figure 4.6.g), suggesting that when expressed under physiologic conditions, HO-1 limits the accumulation of ROS in EC. Fe chelation by DFO reduced intracellular ROS in *Hmox1*$^{-/-}$ EC to levels similar to those of *Hmox1*$^{+/+}$ EC (see Figure 4.6.g). This suggests that the higher levels of intracellular ROS in *Hmox1*$^{-/-}$ EC is due to the higher levels of intracellular labile Fe in these cells (see Figure 4.1.d). Similar results were observed using another Fe chelator, i.e., SIH (data not shown). TNF failed to increase ROS levels in EC as compared with untreated EC (see Figure 4.6.g).

If HO-1 inhibits RelA activity by disabling labile Fe from promoting the generation of ROS, then an antioxidant such as *N*-acetylcysteine (NAC) should mimic the inhibitory effects of HO-1 and/or that of Fe chelation. In keeping with this notion, NAC inhibited by ~50% RelA activity as assessed in EC transiently co-transfected with RelA plus NF-κB luciferase reporter (see Figure 4.6.h).

8. Fe chelation targets the N-terminal domain of RelA

We used a series of chimeric DNA constructs encoding the N- or C-terminal domains from RelA that allowed us to test whether Fe chelation inhibits RelA activity via a mechanism that targets its N- and/or C-terminal domains (285). The chimeric constructs were transiently co-transfected in EC with a NF-κB luciferase reporter derived from the

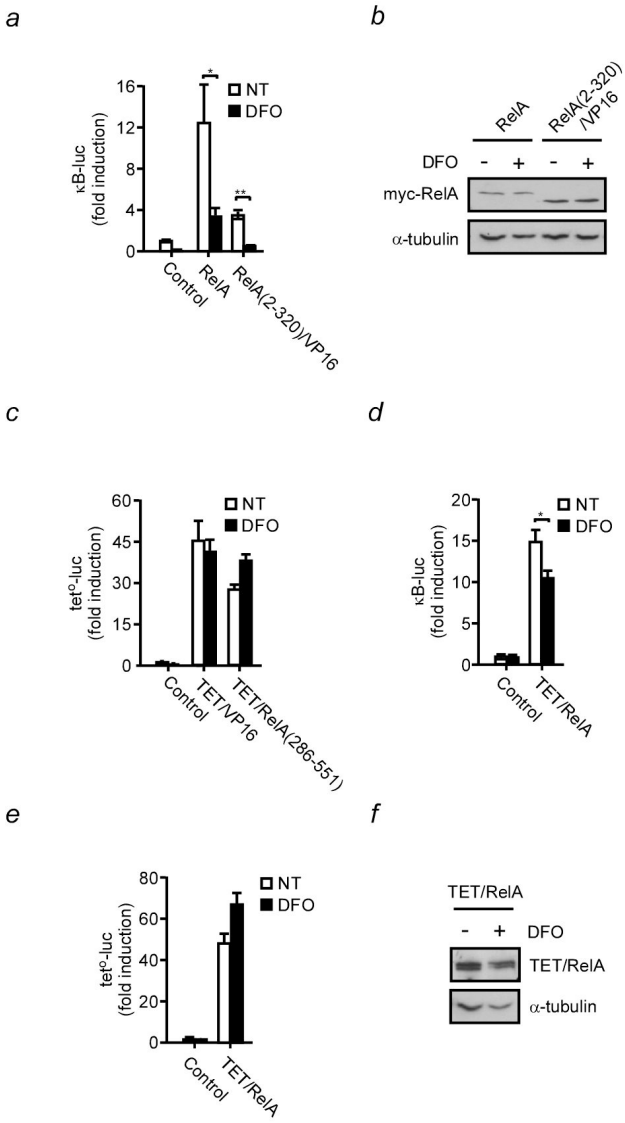


Figure 4.7. Fe chelation modulates RelA transcriptional activity via a mechanism that targets the N-terminal domain of RelA. a) BAEC were transiently transfected with an NF-κB luciferase reporter derived from the porcine E-selectin promoter (κB-luc)(43) plus an expression vector encoding the full length RelA or the N-terminal domain of RelA (aa2-320) fused to the VP16 TAD (RelA(2-320)/VP16). EC were not further treated (NT) or treated with DFO (250 μM, 16h). Shown is mean luciferase normalized to β-galactosidase units ± standard deviation (triplicate sample in one out of three independent experiments). **b)** BAEC were transiently co-transfected and treated as in (a) and over-expressed RelA and RelA (2-320) /VP16 were detected by

western blot using an anti-c-myc antibody, recognizing the c-myc tag in the N-terminus of these constructs. **c)** BAEC were transiently transfected with teto-luciferase reporter (teto-luc) alone (control), co-transfected with teto-luc plus TET/VP16 or a construct encoding a TET DNA binding domain fused to the RelA TAD (TET/RelA(268-551). EC were treated as in (a). **d)** BAEC were transiently transfected with NF-κB luciferase reporter (κB-luc) alone (Control) or co-transfected with κB-luc (same as in (a)) plus an expression vector encoding the TET DNA binding domain fused to the full length RelA (TET/RelA). EC were treated as in (a) and (b). **e)** BAEC were transiently transfected with teto-luciferase reporter (teto-luc) alone (control) or co-transfected with teto-luc plus TET/RelA and treated as in (a-c). Data illustrated in (a-e) are mean luciferase normalized to β-galactosidase units ± standard deviation (triplicate sample in one out of three independent experiments). **f)** BAEC were transiently transfected and treated as in (e) and TET/RelA was detected by western blot using an anti-RelA antibody. *p<0.05.

porcine *E-selectin* promoter (284). DFO inhibited by 83% the transcriptional activity of a chimeric construct in which the N-terminal domain of RelA was fused to a VP16 TAD (RelA (2–320)/VP16), as compared with control EC not treated with DFO (see Figure 4.7.a). This inhibitory effect was similar to the one exerted over the full length RelA (see Figure 4.7.a). Fe chelation did not modulate the expression of RelA (2–320)/VP16 as assessed by Western blot analysis (see Figure 4.7.b). Because RelA (2–320)/VP16 can bind DNA κB sequences via the N-terminal domain of RelA and promote gene transcription via the VP16 TAD, these data suggest that Fe chelation targets the N-terminal domain of RelA.

DFO failed to inhibit the transcriptional activity of a chimeric construct consisting of tet° binding protein TET in its N-terminal portion fused to the C-terminal portion of RelA (TET/RelA (286–551)), as assessed using a tetracycline operon luciferase reporter (tet°-luc) (see Figure 4.7.c). DFO also failed to suppress tet°-luc transcription driven by a chimeric construct consisting of TET fused to VP16 (see Figure 4.7.c).

This suggests that Fe chelation does not target the C-terminal domain of RelA.

We then asked whether the inhibition of RelA transcriptional activity by Fe chelation targets DNA binding via the RHD. To this aim, we made use of a chimeric DNA construct in which TET was fused via its C-terminal domain to RelA. This chimeric protein (TET/RelA) can transcribe a NF-κB luciferase reporter via DNA binding through the RelA RHD (see Figure 4.7.d). The same chimeric protein (TET/RelA) can also transcribe a tet° luciferase reporter via DNA binding through the TET domain (see Figure 4.7.e). Fe chelation by DFO inhibited by 30% TET/RelA-driven NF-κB reporter activity (see Figure 4.7.d) but failed to inhibit TET/RelA-driven tet° reporter activity (see Figure 4.7.e). DFO had no effect on TET/RelA expression as assessed by Western blot analysis (see Figure 4.7.f). Taken together, these data suggest that Fe chelation inhibits RelA activity via a mechanism that involves the recognition of κB DNA motifs by the RHD.

9. Fe chelation targets S276 and S205 in the N-terminal domain of RelA

We hypothesized that Fe chelation might target one or several phospho-acceptors in the N-terminal domain of RelA, an effect that would be consistent with the observed inhibition of RelA activity (86, 219, 285). We tested this hypothesis specifically for the phospho-acceptors S205, S276, S281, and S311. EC were transiently co-transfected with wild-type RelA or with RelA S205A, S276A, S281A, and S311A point mutants plus an NF-κB luciferase reporter derived from the porcine *E-selectin* promoter (284). DFO inhibited by 53 and 43% wild-type RelA and S311A RelA-driven transactivation, respectively, as compared with control EC not treated with DFO (see Figure 4.8.a). This suggests that Fe chelation

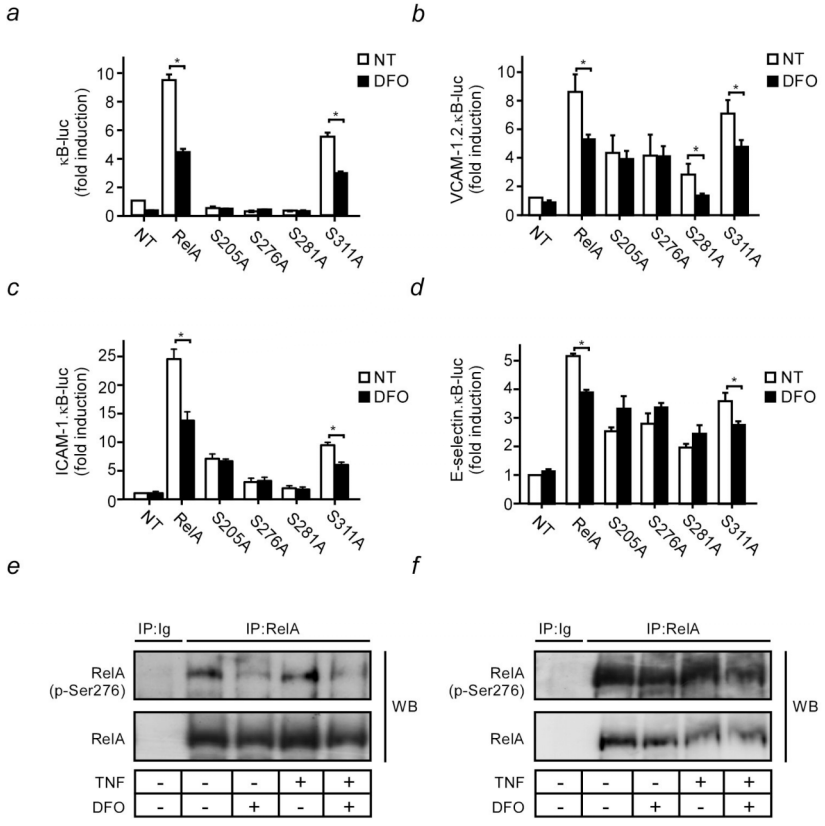


Figure 4.8. Fe chelation modulates RelA transcriptional activity via S205 and S276. BAEC were transiently co-transfected with RelA or RelA S205A, S276A, S281A or S311A point mutants plus an NF-κB luciferase reporter derived from the **a)** porcine E-selectin (κB-luc)(43), **b)** human VCAM-1 (VCAM-1.2-κB-luc)(12), **c)** human ICAM-1 (ICAM-1-κB-luc)(12) or **d)** human E-selectin (E-selectin-κB-luc)(12) promoters. EC were either not further treated (NT) or treated with DFO (250 μM, 24-48h). The data illustrated in (a-d) represents mean luciferase normalized to β-galactosidase units ± standard deviation (triplicate in one out of at least two independent experiments for each promoter used). **e and f)** Confluent MEF (e) or MEC (f) were either left untreated (-) or treated (+) with DFO (250 μM, 16h) and not further treated (-) or treated (+) with TNF (50 ng/ml, 15 min). RelA was immunoprecipitated (IP), total RelA and RelA Ser276 phosphorylation were detected by western blot (WB). Immunoprecipitation with irrelevant Ig was performed as a negative control. *p<0.05.

does not act via S311 to suppress RelA activity. Because the transcriptional activity of the RelA S205A, S276A, and S281A point mutants is abolished using this NF-κB reporter (see Figure 4.8.a) (219), we made use of synthetic NF-κB reporters consisting of two tandem decameric κB sites derived from the human *Vcam-1* (VCAM-1.2-κB-luc), *E-selectin* (E-selectin-κB-luc), or *Icam-1* (ICAM-1-κB-luc) promoters (219).

Fe chelation inhibited by 38% the transcription of VCAM-1.2-κB-luc driven by wild-type RelA. It also inhibited by 52 and 32% transcription driven by the RelA S281A or S311A point mutants, respectively (see Figure 4.8.b), while it failed to inhibit RelA S205A- or S276A-driven transcription (see Figure 4.8.b). Similar results were obtained using the ICAM-1-κB-luc reporter; i.e., Fe chelation inhibited by 53% transactivation by wild-type RelA but failed to inhibit transactivation by RelA S205A or S276A point mutants (see Figure 4.8.c). Fe chelation inhibited by 38% the transactivation of ICAM-1-κB-luc by the S311A RelA mutant while RelA S281A activity was abolished using this reporter (see Figure 4.8.c). Similarly, Fe chelation inhibited by 25% the transactivation of the E-selectin-κB-luc reporter by wild-type RelA (see Figure 4.8.d), consistent with our previous observation that HO-1 and/or Fe chelation are less efficient in blocking E-selectin as compared with VCAM-1 expression (321). Again, Fe chelation failed to suppress S205A or S276A RelA-driven E-selectin-κB-luc reporter transactivation (see Figure 4.8.d). Taken together, these data suggest that Fe chelation inhibits RelA activity via a mechanism that targets specifically RelA S205 and S276 but not S281 or S311.

We then asked whether Fe chelation modulates RelA phosphorylation at S276, an effect that would justify its inhibitory effect (see Figure 4.8.a–d) (219, 285). As described previously (285), RelA

S276 is constitutively phosphorylated in resting MEF (see Figure 4.8.e) as well as in MEC (see Figure 4.8.f). Exposure to TNF failed to increase RelA S276 phosphorylation in MEF (see Figure 4.8.e) but did so in MEC (see Figure 4.8.f). Fe chelation by DFO decreased S276 phosphorylation by 50–60% in resting as well as in TNF-stimulated MEF (see Figure 4.8.e). Fe chelation fully suppressed TNF-driven S276 phosphorylation in MEC (see Figure 4.8.f) while not affecting basal RelA S276 phosphorylation (see Figure 4.8.f). Taken together, these observations suggest that down-modulation of labile Fe inhibits RelA S276 phosphorylation, thus explaining its ability to suppress RelA activity (219, 285).

IV. Discussion

Given that HO-1 overexpression reduces cellular Fe content (328) we asked whether HO-1 would act in a similar manner when expressed under physiologic conditions in EC. Our data suggest that this is the case, because labile Fe content was significantly higher in EC isolated from *Hmox1*$^{-/-}$ mice as compared with those isolated from *Hmox1*$^{+/+}$ mice (see Figure 4.1.d). Based on the above, we suggest that HO-1 might act as a physiologic regulator of EC labile Fe content, a notion in keeping with the deposition of labile Fe in the vasculature of *Hmox1*-deficient mice (70) as well as that associated with the one reported case of *HMOX1* deficiency in humans (71).

Several mechanisms have been proposed as to how HO-1 modulates cellular labile Fe content, including through the up-regulation of FtH (54) and/or the activation of ATPase Fe efflux pumps (328). Given that free Fe can induce the expression of FtH, one would predict that increased labile Fe content in *Hmox1*$^{-/-}$ EC would lead to higher FtH expression (54). However, FtH expression was very significantly

decreased in *Hmox1*$^{-/-}$ vs Hmox1$^{+/+}$ EC (data not shown). One possible explanation for this apparently contradictory observation would be that the accumulation of ROS in *Hmox1*$^{-/-}$ (see Figure 4.6.g) might promote FtH degradation (335), an effect that could account for an overall decrease in FtH expression and thus for the increased labile Fe content observed in *Hmox1*$^{-/-}$ EC (see Figure 4.1.d).

EC from *Hmox1*$^{-/-}$ mice expressed higher basal levels of VCAM-1 as compared with EC from *Hmox1*$^{+/+}$ mice (see Figures 4.1.e–g). This suggests that HO-1 is a physiologic regulator of VCAM-1 expression in EC. Increased VCAM-1 expression in *Hmox1*$^{-/-}$ EC was reversed by Fe chelation (see Figure 4.1.g), suggesting that there is a functional link between the ability of HO-1 to down-regulate labile Fe and to inhibit VCAM-1 expression.

HO-1 overexpression in EC decreases labile Fe content to the same extent as Fe chelation by DFO (see Figure 4.2.a), an effect that probably explains the ability of HO-1 to inhibit the up-regulation of VCAM-1 expression in response to proinflammatory agonists such as TNF. That labile Fe may be involved in the regulation of proinflammatory gene expression in response to TNF is suggested by the observation that TNF triggers a transient rise of labile Fe content in EC (see Figure 4.2.b). When labile Fe is decreased, either by overexpressing HO-1 or by chelating Fe, the ability of TNF to induce the expression of VCAM-1 is inhibited (see Figures 4.2.c–f), a finding in keeping with our previous results (321). The physiologic relevance of these findings is supported by the observation that *Hmox1* deletion increases TNF-driven VCAM-1 expression, an effect reversed when labile Fe is chelated by DFO (see Figure 4.2.g). Similar effects were observed for other adhesion molecules such as E-selectin (see Figure 4.2.h) and ICAM-1 (see Figure 4.2.i), suggesting that the inhibitory effects of HO-1/Fe chelation can be extended to other proinflammatory genes associated with EC activation.

The observation that Fe chelation inhibits TNF-driven *Vcam-1* (see Figures 4.3.a and 4.3.b), *E-selectin* (see Figures 4.3.c and 4.3.d), and *Icam-1* (see Figures 4.3.e and 4.3.f) transcription suggests that NF-κB, a transcription factor required for TNF-driven transcription of these genes in EC (301, 312, 321), might be the target of Fe chelation (321). Although our data suggest that this is the case (see Figure 4.4.a–d), we found that inhibition of NF-κB does not occur at the level of IκBα phosphorylation/degradation (see Figure 4.5.a), C-terminal RelA phosphorylation, i.e. S536 (see Figure 4.5.a), or RelA nuclear translocation (see Figure 4.5.b), and most probably not via the inhibition of NF-κB binding to κB DNA binding motifs in the promoter of *Vcam-1*, *E-selectin*, or *Icam-1* (see Figure 4.5.d). Fe chelation inhibits RelA transcriptional activity via a mechanism that targets specifically its N-terminal domain (see Figures 4.7.a and 4.7.b).

Because N-terminal phosphorylation is required to support RelA transactivation activity (219, 285), we tested whether Fe chelation inhibited RelA activity via a mechanism dependent on S phospho-acceptors in the N-terminal domain of RelA. Fe chelation failed to inhibit the transcriptional activity of RelA S205A or S276A point mutants as assessed by using synthetic NF-κB luciferase reporters containing κB DNA motifs from the human *Vcam-1* (see Figure 4.8.b), *Icam-1* (see Figure 4.8.c), or *E-selectin* (see Figure 4.8.d) promoters. This led to the hypothesis that HO-1 and/or Fe chelation might interfere with S276 phosphorylation. We found that Fe chelation inhibits RelA S276 phosphorylation (see Figure 4.8.e and 4.8.f) in a manner that should account for its inhibitory effect over RelA activity (219, 285). Whether Fe chelation modulates RelA S205 phosphorylation could not be established, as reagents are not available to monitor this phospho-acceptor.

A likely scenario to explain the inhibitory effects observed would be that labile Fe availability, which promotes the generation of ROS (see Fig 4.6.g) via the Fenton reaction (330), might regulate the expression and/or activity of kinase(s) and/or phosphatase(s) that target RelA at S276 and/or S205 (see Figure 4.8.a–d) and thus regulate RelA activity (318). The notion that labile Fe targets RelA activity via ROS is supported by the observation that the antioxidant NAC, a radical scavenger and glutathione precursor, suppressed RelA activity in a manner that mimicked Fe chelation (see Figure 4.6.h). This inhibitory effect is in keeping with our previous observation that BR, another potent antioxidant (53), also inhibits NF-κB activation in EC (321). Whether BR targets RelA in a similar manner to Fe chelation, i.e., inhibiting RelA S276 phosphorylation, remains to be established but is likely to be the case.

Putative candidate kinases modulated by ROS and targeting RelA activity in EC include PKA (180, 182), MSK1 (185), and PKCζ (219, 285). Among these, PKA, which has been shown to target RelA S276, is probably the most likely candidate because Fe chelation can regulate its expression/activity (336). Whether the ability of HO-1 and/or Fe chelation to inhibit RelA activity acts via PKA remains to be established.

We suggest, that the putative kinase(s) and/or phosphatase(s) that target RelA S276 interfere with the mechanism via which the RHD promotes transcriptional activity upon binding to κB DNA motifs. This is supported by the observation that Fe chelation inhibits the transcriptional activity of a RelA chimeric protein (TET/RelA) only when this protein binds DNA via the RHD (i.e., binding to κB DNA recognition motifs in a NF-κB reporter; Figure 4.7.d) but not when the same protein binds DNA via its TET domain (i.e., binding to tet° recognition motifs in a Tet

reporter) (see Figure 4.7.e). It is possible that such kinase(s) and/or phosphatase(s) might target RHD after binding to κB DNA recognition motifs. This hypothesis remains, however, to be tested.

The mechanism via which HO-1 controls NF-κB activation in EC provides clues as to its protective effect in the context of vascular inflammation (337-339). We suggest that down-regulation of EC labile Fe content may inhibit specifically the expression of proinflammatory genes associated with EC activation, e.g., adhesion molecules, while sparing that of anti-apoptotic genes (340). Such an effect is consistent with targeting of RelA S276, a phospho-acceptor that controls RelA *cis*-acting element specificity (219). That is, by down modulating S276 RelA phosphorylation, HO-1 and/or Fe chelation might direct this NF-κB family member to transcribe specific subsets of genes (219). It is likely that this would occur via modulation of RelA interaction with co-activators required to support the transcription of specific gene subsets (318). Such a mechanism would explain how HO-1 has reached a "functional compromise" in which it inhibits specifically the transactivation of proinflammatory genes but probably not that of anti-apoptotic genes required to support its cytoprotective effects, i.e., A1 and c-inhibitor of apoptosis (IAP)2 (340). These two biologic effects, i.e., anti-inflammatory and cytoprotective, are accomplished by different end products of heme degradation, i.e., labile Fe controls NF-κB while CO is cytoprotective without interfering with NF-κB (321).

In conclusion, the effects reported hereby provide clues as to our understanding of the protective effect of HO-1 in countering the pathogenesis of inflammatory diseases, including autoimmune neuroinflammation (339) as well as neuroinflammation emanating from unfettered responses to microbial challenge, e.g., cerebral malaria (338). Our present data support the notion that these protective effects are

mediated, at least partially, via the ability of HO-1 to modulate EC labile Fe content, an effect that regulates NF-κB activity in a manner that is anti-inflammatory and cytoprotective. Finally, these data might also help in providing a mechanism for the palliative effects of Fe chelation therapies.

V. Materials and Methods

Mice

BALB/c *Hmox1*$^{-/-}$ mice, generated by S.-F. Yet of the Pulmonary and Critical Care Division, Brigham and Women's Hospital (341), were maintained at the Instituto Gulbenkian de Ciência (Oeiras, Portugal) under specific pathogen-free conditions in accordance with guidelines from the Animal Use and Institutional Ethical Committee of this institute. Genotypes were verified by genomic PCR using the following primers: *Hmox1* (5'-GGT GAC AGA AGA GGC TAA G-3' and 5'-CTG TAA CTC CAC CTC CAA C-3') and neomycin (5'-TCT TGA CGA GTT CTT CTG AG-3' and 5'-ACG AAG TGA CGC CAT CTG T-3').

Cell culture

Murine endothelial cells (MEC) were isolated as described (342). Briefly, three hearts were mechanically minced, digested (2.2U/ml collagenase A at 37°C for 30 min; Sigma-Aldrich), washed, and labeled with rat anti-mouse PECAM-1 Ab (CD31; BD Pharmingen) plus goat anti-rat IgG microbeads MACS (Miltenyi Biotec). Labeled EC were purified in magnetic separation columns as per the manufacturer's instructions (Miltenyi Biotec). EC phenotype was assessed by cobblestone morphology and confirmed by flow cytometry (CD31/CD105 expression). MEC were passaged 1/3 and used between passages 6 and 12. Bovine aortic endothelial cells (BAEC) were obtained as previously described

(321) or purchased from Vec Technologies or Cell Systems and cultured on gelatinized plates in complete medium (MCDB-131; 20% FCS, 20 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin; Invitrogen Life Technologies). BAEC were passaged 1/3 and used between passages 6 and 10. Immortalized human epithelial kidney 293 (HEK-293) cells were obtained from American Type Culture Collection and cultured as for BAEC. Pooled HUVEC (Clonetics) were cultured in EBM-2 medium supplemented according to the provider's instructions (Clonetics). HUVEC were passaged 1/3 and used between passages 4 and 6. Mouse embryonic fibroblasts (MEF) from *RelA*$^{-/-}$ mice were provided by Dr. A. Beg from Columbia University, New York, NY (343). MEF were cultured in DMEM (Mediatech) supplemented with FCS (10%), penicillin (50 U/ml), and streptomycin (50 µg/ml) (Atlanta Biologicals). *RelA*$^{-/-}$ MEF were stably transfected with human RelA recombinant retroviruses essentially as described (219).

Immunoprecipitation

Cells were lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 1% Triton X-100, and a Roche Protease Inhibitor Cocktail (complete EDTA-free, one tablet per 10 ml of lysis solution). Soluble fractions were incubated with a protein G-Sepharose bead/antibody complex in borate-buffered solution with 1% Triton X-100 (for 4 h at 4°C). RelA was immunoprecipitated with anti-mouse mAb (2 µg; catalog no. SC-8008, Santa Cruz Biotechnology). Immunoprecipitated proteins were washed in lysis buffer, resuspended in 2x sample buffer, heated to 100°C for 5 min, resolved by SDS-PAGE, and subjected to Western blotting.

Western blots

Cells were lysed in Laemmli sample buffer and proteins were resolved

by electrophoresis on 8–10% SDS-polyacrylamide gels (344), transferred to polyvinylidene fluoride membrane (Bio-Rad), and blotted with Abs to HO-1 (catalog no. SPA-895 or SPA-896, Stressgen), RelA (catalog no. SC-372, Santa Cruz Biotechnology), phospho-S536-RelA (catalog no.3031, Cell Signaling), phospho-S276-RelA (catalog no.3037, Cell Signaling), IκBα (catalog no. SC-371, Santa Cruz Biotechnology), phospho-S^{32}-S^{36}-IκBα (catalog no.9246, Cell Signaling), c-Myc (clone 9E10; American Type Culture Collection), or α-tubulin (clone DM 1A; catalog no. T-9026, Sigma-Aldrich). Primary Abs were detected using HRP-conjugated secondary Abs (Pierce). Labeling was visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to BioMax film (Kodak).

Flow cytometry

Confluent EC (~4 x 10^5) were washed in PBS and detached with 0.125% trypsin/0.05% EDTA (at 37°C for 5 min; Invitrogen Life Technologies). Digestion was stopped with complete MCDB-131 medium and cells were pelleted by centrifugation (200 x*g* at 4°C for 3min), resuspended in 150µl of FACS medium (PBS, 3% FCS, and 0.01% sodium azide), transferred into round-bottom 96-well plates (TPP) and pelleted (200 x*g* at 4°C for 3 min). Supernatant was removed and cells were incubated in 25–50 µl of FACS medium with primary Ab (5 µg/ml for 30 min at 4°C) against endoglin/CD105 (catalog no.550546, BD Biosciences), PECAM/CD31 (catalog no.553370, BD Biosciences), or VCAM-1/CD106 (catalog no.553331, BD Biosciences). Cells were washed in FACS medium (3x 200 µl at 200 x*g* and 4°C for 3 min) and primary Abs were detected using FITC-labeled secondary Abs (catalog no. F-6258, Sigma-Aldrich) in FACS medium (25 µl for 30 min at 4°C). Cells were costained with propidium iodide (0.5µg/ml) to assess cellular viability. Fluorescence was

measured by flow cytometry on a FACSCalibur device (BD Biosciences).

Cellular Fe assay

Labile and non-labile Fe were measured essentially as described previously (345) using Ferene-S (346). Briefly, confluent EC (1 x 10^7) were harvested by trypsinization, pelleted (200 x*g* at 4°C for 3 min), washed three times with Fe-depleted PBS (1% Chelex-100 for 12 h) (Bio-Rad), and digested (0.5 ml of 25% perchloric acid at 4°C for 30 min). Supernatants (containing labile Fe) were collected after centrifugation (10,000 x*g* at 4°C for 15 min), pellets were digested (0.1 ml of 10 M nitric acid at 60°C for 16 h), pH was neutralized (10 M NaOH), cellular debris was pelleted (10000 x*g* at 4°C for 15 min), and supernatants were collected as the non-labile cellular Fe fraction. Fe in both fractions was converted to the ferrous (Fe^{2+}) form using sodium ascorbate (0.25 M, 0.1 ml) plus an equal volume of ammonium acetate (40%). Ferene-S (600 µM; BioVectra) was added and OD was measured at λ_{594} nm. Fe levels were normalized to total protein content as determined by Bradford assay. Fe fractions are expressed as moles of Fe per milligram of cellular protein. Assays were performed at least in triplicate for each condition.

Quantitative real-time PCR

cDNA was obtained from total RNA as described (347). The relative number of mRNA transcripts was assessed using LightCycler real-time quantitative PCR (Roche) using FastStart DNA SYBR Green I mix (Roche). The transcript number was calculated using a $2^{-\Delta CT}$ method (relative number) (348) or quantified as an absolute value using standard cDNA plasmids encoding the same sequences as the ones amplified by PCR. Results were normalized to hypoxanthine-guanine-phosphoribosyltransferase (HPRT) (349). PCR primers used were as

follows: 5'-TGG GAG AGA CAA AGC AGA AG-3' and 5'-AGA TGG TCA AAG GGA TAC AC-3' or 5'-TGC CGA GCT AAA TTA CAC ATT G-3' and 5'-CCT TGT GGA GGG ATG TAC AGA-3' for *Vcam-1*; 5'-GCC TTG GTA GAG GTG ACT GAG-3' and 5'-GAC CGG AGC TGA AAA GTT GTA-3' for *Icam-1*; 5'-CTG CTC TTG TTT TTG TTC TC-3' and 5'-CTT CCA TAG TCA GGG TGT TC-3' for *E-selectin*; and 5'-GCT GGT GAA AAG GAC CTC T-3' and 5'-CAC AGG ACT AGA ACA CCT GC-3' for *Hprt*.

Reporters and expression constructs

Rattus norvegicus Hmox1 cDNA was expressed in pcDNA3 (Invitrogen Life Technologies) (58). N-terminal Myc-tagged RelA, p50, and cRel expression vectors have been described in Refs. (285) and (340), respectively. The NF-κB luciferase (luc) reporter (κB-luc) consists of three NF-κB binding sites, i.e., NF-E-selectin, κB-2, and κB-3, derived from the porcine E-selectin promoter (284). Other NF-κB luciferase reporter constructs, i.e., VCAM-1.2-κB-luc, ICAM-1-κB-luc, and E-selectin-κB-luc have been described elsewhere (219). Briefly, the minimal SV40 promoter in the pGL3-promoter vector (Promega) was replaced with a minimal 5'-GAT CTG GGT ATA TAA TGG ATC CCC GGG TAC GCA GCT CAA GCT-3' promoter, and the synthetic oligonucleotides coding for the κB consensus sequence derived from the *Vcam-1*, *Icam-1*, or *E-selectin* promoter were cloned upstream of this minimal promoter so that they differ only in their decameric κB consensus sequences. The prototypical sequence used was 5'-GCT-κB-decamer-CTGAGCTCCT-κB-deamer-CTCAGCT-3', in which the decameric κB consensus were GGGATTTCCC for VCAM-1.2-κB-luc, TGGAAATTCC for ICAM-1-κB, and GGGGATTTCC for E-selectin-κB-luc (219). The human VCAM1 (–1716/+119) luciferase reporter was kindly provided by W. C. Aird (Department of Molecular Medicine, Beth

Israel Deaconess Medical Center, Boston, MA) (350). The porcine E-selectin (–1286/+482) luciferase reporter has been described (351). The ICAM-1-luc (–339/-30) luciferase reporter was kindly provided by J. S. Pober (Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut) (352). The tetracycline operon (tet°) luciferase reporter (tet°-luc) contains seven upstream tet° sequences derived from the tet° flanked by two divergently orientated cytomegalovirus minimal promoters driving the full-length firefly luciferase gene (H. Bujard, University of Heidelberg, Germany) (285). N-terminal Myc-tagged RelA and serine to alanine (S205A, S276A, S281A, and S311A) RelA mutants have been described (219). Myc-tagged RelA chimeric expression vectors have been described elsewhere (285). Briefly, RelA (2–320)/VP16 is composed of the RelA RHD (aa 2–320) fused to virion protein 16 (VP16) (285). TET/RelA (aa 2–551) is composed of a tet° DNA binding domain (TET) fused to the full-length RelA (285). TET/RelA (268–551) is composed of TET fused to the RelA transactivation domain (TAD) (aa 268–551) (285). TET/VP16 is composed of TET fused to VP16 (285). The pSV-β-galactosidase reporter (Promega) consists of the *lacZ* gene from *Escherichia coli* under the control of the SV40 early promoter and enhancer, providing for constitutive expression in EC. The pCAGGS-AFP vector drives enhanced GFP expression by a CMV/actin hybrid promoter (353). The pRL-SV40 control vector contains the cDNA encoding *Renilla* luciferase under the control of the SV40 early promoter and enhancer (Promega).

Recombinant adenoviruses

HUVEC were transduced with recombinant adenoviruses as described (321). The recombinant β-galactosidase adenovirus was a gift from R. Gerard (University of Texas Southwest Medical Center, Dallas, TX). The recombinant HO-1 adenovirus containing the entire coding region of the

R. norvegicus HO-1 cDNA has been described (321).

Transient transfection and luciferase assays

BAEC, HUVEC, and HEK-293 cells were transfected at 60–70% confluence using the Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen Life Science Technologies). In BAEC and HEK-293, 3 μg of total DNA was used per well of a 6-well plate with 2 μl of Lipofectamine 2000. For HUVEC, 1.5–2.5 μg of DNA was used with 1 μl of Lipofectamine 2000. The total amount of DNA was kept constant using pcDNA3 (Promega). Briefly, cells were washed and placed in serum and antibiotic-free medium followed by the addition of the DNA/Lipofectamine and replaced thereafter (2.5 h) with medium containing sera. Transfection efficiency was determined by either flow cytometry or fluorescence microscopy in cells co-transfected with pCAGGS-AFP. Transfection efficiency was typically 50% for BAEC, >95% for HEK-293, and 5–20% for HUVEC.

Luciferase assays were performed using a single (firefly) luciferase assay system (Promega) according to manufacturers instructions with a β-galactosidase control expression vector (pSV-β-galactosidase) as the control for transfection and extraction efficiency. β-Galactosidase expression was detected using Galacto-Light Plus β-Galactosidase Reporter Gene Assay System (Applied Biosystems). Alternatively, a Dual Luciferase Assay System (Promega) was used with a *Renilla* control expression vector (pRL-SV40). Assays were performed in triplicate and luciferase values were normalized to either β-galactosidase or *Renilla* luciferase.

Cellular ELISA

Two days postconfluence, HUVEC were exposed to human recombinant

TNF at the indicated concentrations and times. The procedure has been described in detail elsewhere (321).

Immunostaings

Confluent EC were washed in PBS and fixed with 3.7% paraformaldehyde (20 min at room temperature). Immunostainings were performed using an anti-RelA mAb (catalog no. SC-372, Santa-Cruz Biotechnology) plus a FITC-labeled goat anti-rabbit polyclonal Ab (Pierce). DNA was stained using 20 ng/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Stainings were detected using a fluorescence microscope (Leica DMRA2) at $\lambda Ex_{480/40\ nm}$ and $\lambda Em_{527/30\ nm}$ for FITC and $\lambda Ex_{360/40}$ nm and $\lambda Em_{470/40}$ nm(where Ex is excitation and Em is emission) for DAPI. Images were acquired using Metamorph version 4.6r5 software (Universal Imaging Corporation) and analyzed with NIH ImageJ software.

Reagents

Human recombinant TNF (R & D Systems) was resuspended in PBS plus 0.1% BSA and added directly to culture medium. Deferoxamine mesylate (DFO) (Sigma-Aldrich) was dissolved in culture medium at 30 and 250 μM. *N*-(2-hydroxybenzyl)-L-serine (HBSer; 0.5–5 mM) (354) was a gift from R. Tyrrell, Department of Pharmacology, University of Bath, Bath, U.K. (332). Salicylaldehyde isonicotinoylhydrazone (SIH; 5–100 μM) (gift from Dr. P. Ponka, McGill University, Montreal, Quebec, Canada) was prepared as described (333). *N*-acetyl-L-cysteine (Sigma-Aldrich) was dissolved in culture medium, neutralized to pH 7.5 with 0.2–1 M NaOH, and added to culture medium (0.5 and 25 mM). 5-(and-6)-Chloromethyl-2',7'-dichlorofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Molecular Probes) was dissolved in DMSO (Sigma-Aldrich) and added to culture medium (3 μM).

ROS detection

Cellular ROS content was assayed by flow cytometry using CM-H_2DCFDA. Two days postconfluence, MEC were collected by trypsinization, pelleted (200 x*g* at 4°C for 3 min), washed in PBS, and incubated with CM-H_2DCFDA (3 μM in PBS; 37°C for 15 min). Cells were washed twice in PBS with 2% FCS and resuspended in PBS with 2% FCS (37°C for 30 min). Fluorescence was measured by flow cytometry (FACSCalibur; BD Biosciences) and data were analyzed using FlowJo software (Tree Star). Propidium iodide (5 μg/ml) was used to exclude dead cells.

EMSA

Nuclear extracts were obtained from confluent BAEC as described (285, 340). Protein concentration was determined by Bradford assay. EMSA was performed essentially as previously described (285, 340). Briefly, double-stranded oligonucleotides were generated with the following sequences. 5'-AGT TGA GGG GTT TCC CCC AGG C-3' (VCAM-1.1); 5'-AGT TGA GGG GAT TTC CCC AGG C-3' (VCAM-1.2); 5'-AGT TGA GTG GAA ATT CCC AGG C-3' (ICAM-1); 5'-AGT TGA GGG GGA TTT CCC AGG C-3' (E-selectin); and 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (Ig-κB). Oligonucleotides were labeled by phosphorylation with [γ-^{32}P]ATP. EMSA was visualized in the form of autoradiographs indicating DNA/protein binding.

Statistical analysis

Statistical significance was assessed using an unpaired two-tailed *t* test assuming unequal variances as specified in *Results* according to experimental design, except for data in Figure 4.1.e where a one-sample, one-tailed z test was used.

VI. Author contribution and Acknowledgments

NP performed experiments and analysis revealing the effect of HO-1 and DFO on TNF-driven adhesion molecule mRNA expression (see Figure 4.2.g-i) and together with GS identified RelA phospho-acceptors targeted by DFO (see Figure 4.8.a-d) and provided intellectual support.

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CHAPTER V
GENERAL DISCUSSION

1. One transcription factor - many transcriptional responses

Living in an ever-changing environment, cells have evolved numerous mechanisms to sense their environment, communicate with each other and integrate the information allowing for continuous adaptation to the environment. A significant part of this signal integration and decision-making process occurs at the level of regulation of gene transcription (76). While the entire human transcriptional machinery is estimated to consist of 1,000 to 2,000 TFs regulating the expression of ~30,000 genes, little is known about the specificity of their regulatory functions, levels and patterns of their expression and their interactive networks (355). Much of the diversity of transcriptional responses is due to TF binding to DNA regulatory regions that depends largely on post-translational regulatory mechanisms targeting the TF as well as histones embedded by the DNA (215, 356). The NF-κB family of TFs represents one of the best-characterized TF in which post-translational modulations play a central role in the transcription of hundreds of target genes controlling inducible and cell type specific responses that are central to maintain organism integrity. Due to its dominant role in regulating cell survival, proliferation and PCD associated with inflammatory and immune responses, NF-κB has gained a tremendous research interest soon after its discovery and has since served as a model system for understanding basic mechanisms of transcriptional regulation in higher eukaryotes (80).

NF-κB originated at the dawn of the Metazoan (101) serving ever since as a common integrator of diverse environmental cues, triggering specific adaptive responses, which probably contributed to the complexity of mechanisms controlling its transcriptional activity. The central mechanism regulating the activity of this TF, relying on the

degradation of its constitutive inhibitors IκBs, has been traced back to ancient arthropods, such as the horseshoe crab (97). To what extent the other mechanisms responsible for the diversity of NF-κB-dependent responses are evolutionary conserved is a matter of debate. Post-translational modifications probably evolved as an additional mechanism capable of fine-tuning NF-κB activity by regulating different events interfering with its function. These include NF-κB stability, subcellular localization, interaction with IκB, DNA, transcriptional cofactors and chromatin remodeling complexes (176). These different mechanisms of action are best illustrated for RelA, the major transcriptionally active member of the NF-κB family. It is increasingly accepted that the RelA transcriptional activity is not solely regulated by a single post-translational modification. Rather, a combinatorial usage of both activating and inhibiting post-translational modifications in response to a particular stimulus are ensuring the specific temporal and spatial transcriptional rate of a particular RelA-dependant gene. Thus, post-translational modifications should be represented as a sequence of coordinated events leading to the activation or the inhibition of RelA-dependent transcription.

Phosphorylation of RelA S276 is one of the best-characterized post-translational modifications targeting this TF. Following LPS stimulation, PKA-mediated RelA phosphorylation at S276 induces conformational changes promoting the interaction of RelA with the transcriptional activator HAT CBP/p300, while diminishing the binding of transcriptional repressors such as HDACs (182). Besides controlling chromatin accessibility for the transcriptional machinery, the opposing effects of HATs and HDACs also regulate RelA transcriptional activity by targeting different RelA lysine (K) residues for acetylation and deacetylation, respectively (see Chapter I Section II.2.3.2.). Depending

on the K residue, RelA acetylation status influences its transcriptional activity by increasing or decreasing the IκB and/or DNA binding (i.e. K122, K123, K218, K221), regulating other post-translational modifications (i.e. K310) as well as by an unknown mechanism (i.e. K314, K315) (198-200). Several of these K residues can undergo other post-translational modifications such as methylation and ubiquitination. Contrary to RelA K314 and K315 acetylation, which promote NF-κB-dependent transcription, methylation of these K residues decreases NF-κB transcriptional activity by promoting RelA ubiquitination and proteasomal degradation (204). K310 monomethylation contrary to acetylation also inhibits RelA transcriptional activity via a mechanism that involves histone H3 K9 methylation (203). It is therefore possible that acetylation of these residues could promote RelA transcriptional activity by preventing the inhibitory effect associated with their methylation. On the other hand, both K218 and K221 acetylation and methylation promote RelA transcriptional activity, possibly regulating different sets of NF-κB-dependent genes (199, 205). Moreover, K123, K310 and K315 have recently been identified to undergo ubiquitination as well (212). Thus the balance of and the competition between different post-translational modifications rather than a single post-translational modification are likely to be essential in regulating the transcriptional rate of a particular gene in a cell-type- or a stimulus-dependent way.

RelA S276 phosphorylation and its consequent binding to CBP/p300 are critical for RelA transcriptional activity. This regulatory mechanism relies on at least three different kinases targeting cytoplasmic as well as nuclear RelA S276 in response to distinct signaling pathways (see Chapter I Section II.2.3.1.). Moreover, one of these kinases, i.e. PKA, also phosphorylates evolutionary conserved S276 phosphorylation site in *Drosophila*, as illustrated by its effect in

controlling the nuclear translocation of the *Drosophila* RelA homologue Dorsal (177, 189). Phosphorylation of RelA S276 is, however, not globally required for its transcriptional activity, but it is rather dependent on the nature of the DNA sequence to which it is bound, regulating transcription in a promoter specific manner (219). We found that RelA phosphorylation mutant, mimicked by S276A mutation, translocates to PML nuclear bodies, an effect possibly associated with its gene-specific transcriptional activity (see Chapter III Figures 3.1. and 3.2.). While the implications of this phenomenon are to be addressed, there are several possible explanations for RelA translocation into the PML nuclear bodies. PML nuclear bodies can both positively and negatively regulate transcription. RelA translocation into PML nuclear bodies has been previously implicated in the regulation of its transcriptional activity, leading to RelA proteasomal degradation and hence termination of NF-κB-dependent gene transcription (211). While we did not assess the stability of RelA inside the PML nuclear bodies, based on this observation, it is possible to predict that translocation of hypophosphorylated RelA to PML nuclear bodies could precede its degradation. On the other hand, PML nuclear bodies can store accumulated nuclear proteins independently of their degradation (231). RelA S276A mutant decreases the expression of IκBα, promoting RelA nuclear retention (308). In one scenario, RelA S276A sequestration in PML nuclear bodies could induce gene transcription of a RelA S276 phosphorylation state-independent subset of NF-κB dependent gene, such as the major histocompatibility (MHC) class I (219). In this case, PML nuclear bodies could serve as a site of active transcription or by regulating RelA post-translational modifications as shown for other TF (299). In another scenario, PML nuclear bodies could regulate termination of NF-κB activity, serving as a nuclear pool of

transcriptionally inactive RelA in the absence of substantial RelA-IκBs nuclear export, independently of its degradation. It should be noted that RelA phosphorylation at other phospho-acceptor sites such as S468 can mediate its translocation to other nuclear compartments, such as illustrated by the speckled nuclear distribution of RelA in response to TNF (190). This possibly promotes COMMD1-mediated RelA ubiquitination followed by its proteasomal degradation (191).

2. NF-κB in physiology and pathology

The TF NF-κB is critically involved in the regulation of both homeostatic and pathological processes. While a complexity of mechanism regulating NF-κB transcriptional activity have evolved, aimed at re-establishing homeostasis in response to different forms of stress, deregulated NF-κB activity *per se* can lead to a number of pathological conditions including immune-mediated inflammatory diseases. The role of NF-κB in inflammation is complex and the timing of specific NF-κB-dependent gene expression patterns highly correlates with ongoing physiological processes over the course of inflammation (357). Hence, first genes to be expressed facilitate neutrophil infiltration, following the expression of genes facilitating macrophage recruitment to the site of inflammation, with a concomitant expression of genes helping tissue repair and restoration of homeostasis (357). As such, NF-κB-dependent pro-inflammatory responses must be shut down after clearing the injurious stimulus. Still, NF-κB activity is required in what concerns its pro-apoptotic function in neutrophils as well as its anti-inflammatory and anti-apoptotic functions in other cells (358). Supporting this notion, genetic deletion of a major transcriptionally active NF-κB family member RelA is lethal because it promotes PCD in response to pro-inflammatory

agonists such as TNF (108). Thus, the mechanisms controlling NF-κB activity are critical for the resolution of inflammation. One of the mechanisms regulating termination of NF-κB activation probably relies on NF-κB post-translational modifications, which can besides other processes inhibit NF-κB DNA binding as well as promote NF-κB interaction with IκBα, translocation to specific subnuclear compartments or NF-κB degradation (176). This can be achieved via different mechanisms including via the action of stress-responsive genes, such as HO-1 (see Chapter IV). We demonstrate that HO-1 controls RelA phosphorylation at S276, an effect that can itself regulate RelA translocation to specific subnuclear compartments such as PML bodies (see Chapters III, IV). We also show that a member of CCT family of proteins with chaperone activity can regulate RelA transcriptional activity in a promoter-specific context by modulating RelA K122/123 acetylation, and as such contribute to the resolution of inflammation (see Chapter II).

HO-1 is a stress responsive gene that uncouples inflammatory responses from tissue damage and disease (68). This notion is supported by the observation that inflammatory responses fail to restore homeostasis in the absence of HO-1 (339, 359). While there are many mechanisms involved in salutary effects of HO-1, we found that one of these mechanisms in EC probably relies on its ability to control the extent of NF-κB activation. Expression of HO-1 acts directly or indirectly to control cellular labile Fe content, an effect associated with the inhibition of RelA S276 phosphorylation controlling NF-κB activity (see Chapter IV Figures 4.1.d, 4.2.a and 4.8.). If not tightly controlled, cellular labile Fe can be highly deleterious, promoting the generation of ROS (see Chapter IV, Figure 4.6.g) via the Fenton reaction. Along with numerous harmful effects, the oxidative stress associated with the accumulation of ROS can modulate NF-κB-dependent transcription via

different mechanisms (360) including throughout the activation of PKA (180, 182) and MSK1 (185), two kinases targeting RelA S276 for phosphorylation (180, 185). Reducing the accumulation of reactive labile Fe and hence accumulation of ROS could therefore regulate PKA expression/activity and thus a likely scenario to explain regulatory effect of HO-1 over NF-κB activity would be throughout inhibition of PKA-driven RelA S276 phosphorylation, thus inhibiting NF-κB transcriptional activity (see Chapter IV Figure 4.8.). Inhibition of PKA activity, on the other hand, can induce RelA translocation to PML nuclear bodies, an effect presumably associated with inhibition of RelA S276 phoshorylation (see Chapter III Figures 3.1. and 3.3). It would be therefore interesting to assess whether HO-1, by inhibiting RelA S276 phosphorylation, also promotes RelA translocation to specific nuclear compartments such as PML nuclear bodies. While PML nuclear bodies can regulate the activity of TF by regulating its post-translational modifications as well as availability of components of the transcriptional machinery (231), translocation of RelA into PML nuclear bodies could be associated with the gene-restricted transcriptional activity of differentially phosphorylated S276 RelA (219). This mechanism could possibly underlie the inhibition of proinflammatory gene expression associated with the HO-1 expression in EC (see Chapter IV).

The specific environmental conditions have likely shaped the mechanisms underlying a particular RelA post-translational modification dictating a specific gene expression pattern required for an adequate response to such condition. As such, S276 phosphorylation can be induced in a response to a wide range of environmental conditions (176), selectively regulating the expression of a subset of NF-κB-dependent genes (219). While we did not assess the effect of HO-1 on a broad array of RelA-dependent genes, this could possibly explain how HO-1

has reached a "functional compromise" in which it inhibits specifically the transactivation of proinflammatory genes but probably not that of cytoprotective genes, some of which are required to support the cytoprotective effects of HO-1, i.e. A1 and c-inhibitor of apoptosis (IAP) 2 (340).

Given a long history of HO-1 and NF-κB co-existence and the number of environmental conditions linked with the expression of both NF-κB and HO-1, it is not surprising that one of the HO-1 protective effects that has evolved is to inhibit RelA S276 phosphorylation. Other stress-responsive genes have been shown to reduce NF-κB activation including several NF-κB-dependent genes, e.g. A20, IκBs, serving as a negative feedback loop aiming in termination of NF-κB-dependent transcription (150, 151, 361). Thus controlling the HO-1 activity could be a valuable therapeutic target in several inflammatory pathological conditions associated with increased RelA S276 phosphorylation such as polymicrobial infection-induced lung inflammation (362) and others (363, 364). Indeed, other anti-inflammatory drugs exert their effects by inhibiting RelA S276 phosphorylation (365).

Persistent NF-κB activation has been linked with the initiation and progression of tumor formation, an effect associated with sustained, inflammatory cell infiltration and production of cytokines, chemokines and growth factors as well as cell proliferation and survival (85). The role of RelA S276 phosphorylation in these oncogenic processes has been recently suggested as well. PKA-mediated RelA S276 phosphorylation contributes to the malignant phenotype of head and neck squamous cell carcinoma (HNSCC) (366). Given that HO-1 can inhibit RelA S276 phosphorylation, acting as an anti-inflammatory gene, this could possibly be added on the list of mechanisms affording the anti-tumorogenic role of HO-1. Supporting this notion, other anti-inflammatory drugs can stop

tumor progression including colorectal cancer (367), an effect associated with the nucleolar RelA translocation, inhibition of NF-κB-dependent transcription and the induction of PCD (234). We have shown that hypophosphorylated S276 RelA translocates to PML nuclear bodies. As RelA translocation to PML nuclear bodies can induce RelA ubiquitination and subsequent proteasomal degradation (211), this could possibly serve as a mechanism by which HO-1 could exert its anti-tumorogenic function in the context of the inflammation. On the other hand, however, it is important to note that HO-1 is a cytoprotective gene and its prolonged activity could also promote tumor formation. Indeed, HO-1 activity has been associated with several cancer formations, i.e. lymphosarcoma, prostate cancers, pancreatic carcinoma and squamous carcinoma (368).

Despite the extensive research about NF-κB transcriptional regulation, much remains unknown. One goal that would bring us closer to understanding the complexity of NF-κB-driven responses is to identify novel regulators of NF-κB transcriptional activity. To that aim we have performed an RNAi screen and identified the chaperonin CCT as an evolutionary conserved regulator of NF-κB transcriptional activity that exhibits its effect by modulating RelA acetylation (see Chapter II Figures 2.1. and 2.5.a). CCT controls RelA activity in a promoter-specific context, depending on kinetics of the NF-κB transcriptional response. While promoting transcription during the early phase of RelA activation, CCT diminishes gene expression during the late phase of RelA transcriptional activity (see Chapter II, Figure 2.2.). Supporting this, other genes, such as COX2, that promote inflammation during the initiation stages by producing pro-inflammatory mediators, e.g. PGE_2, can switch their action towards anti-inflammatory, e.g. production of 15-deoxy-Δ12,14-PGJ_2, promoting the resolution of inflammation. (see Chapter I Section I).

Whereas acetylation can affect RelA activity on different levels, our data suggests that during the late phase of RelA activation, CCT acts via a mechanism that targets specifically RelA K122/123 acetylation, an effect associated with decreased DNA binding and termination of NF-κB-dependent gene expression (see Chapter II Figures 2.4. and 2.5.d) (198). Understanding of the mechanisms underlying RelA acetylation under physiological conditions is still very limited. While acetylation can either increase or decrease levels of transcription, due to the lack of appropriate approaches and means available to date, increased RelA acetylation is generally associated with a concomitant increase in NF-κB activity and prognosis of immune-mediated inflammatory diseases such as associated with *Haemophilus influenzae* infection (369) or chronic obstructive pulmonary disease (COPD) (370). On the other hand, RelA acetylation can also be associated with the expression of anti-inflammatory NF-κB-dependent genes such as IL-10 in response to infection by *Mycobacterium tuberculosis* or *leprae*, *Candida albicans* or viruses including *Morbilliviruses*, i.e. *Measles virus*, as well as the human immunodeficiency virus-1 (HIV), (371).

CCT was considered as an exclusive mediator of actine and tubuline folding with its effects on other proteins folding being appreciated only recently (see Chapter I Section III.2.). It is therefore not surprising that the role of CCT in disease is largely unknown and it is mainly associated with “misfolding” diseases, which include besides others Alzheimer’s, Parkinson’s and Huntington’s disease (372). Physiological roles of CCT also include cell cycle progression, cytoskeletal protein polymerization and fibroblast motility and contractility (74, 75), processes involved in tissue repair responses and resolution of inflammation. At the same time proper termination of NF-κB activity is critical for the resolution of inflammation and return to homeostasis. We

hereby show that CCT regulates NF-κB-mediated transcription, diminishing RelA DNA binding via a mechanism targeting specifically RelA K122/123 acetylation during the late phase of NF-κB transcriptional activity. We infer that such an effect would be consistent with CCT role in resolution of inflammation (see Chapter II). Furthermore, we found that CCT controls the acetylation of endogenous CBP (see Chapter II Figure 2.5.b), pointing at reduced HAT activity. While CBP is the best-described RelA acetyl transferase, targeting all the K residues interfering with NF-κB-driven transcription, the effect of CCT seems to be rather specific towards K122/123 acetylation (see Chapter II Figure 2.5.d). Additional studies will be necessary to address the exact mechanism by which CCT regulates NF-κB activity.

3. NF-κB beyond comprehension

Being central to homeostasis, NF-κB family of transcription factors has gained the attention of scientists like few others. While there are more than 30,000 questions answered about its role in physiological and pathological conditions, structure and function, ancient origin and evolutionary conservancy and mechanism regulating its activity, every novel insight into the complexity of NF-κB signaling system, however, opens many questions that would be of interest to address. One such question is indisputably the selectivity of NF-κB transcriptional responses. While post-translational modifications probably play a central role in fine-tuning NF-κB transcriptional activity, the complexity and variety of distinct functional outcomes underlying these modifications in a cell- and stimulus-specific manner are proving difficult to reveal their dynamic interaction networks under the physiological conditions (176). Moreover, several NF-κB family members as well as functional

outcomes underlying NF-κB post-translational modifications are highly redundant in mammalian cells. On the other hand, different NF-κB family members as well as the signal transduction pathways leading to its activation are highly evolutionary conserved. Thus, revealing mechanisms underlying NF-κB post-translational modifications in simpler organisms and using evolutionary comparison could possibly provide clues as to our understanding of the importance of a particular post-translational modification in controlling NF-κB transcriptional activity. While little is known about the role of post-translational modifications regulating NF-κB transcriptional activity throughout evolution, it is becoming clear that post-translational modifications are widespread from archaebacteria to humans, increasing protein functional complexity when rapid and/or profound responses to environmental changes are required (373). Comparative analysis of NF-κB RHD domains among different species shows high conservation of several RelA residues undergoing post-translational modifications, i.e. K56, K79, K122, K123, S205, K218, K221, T254, S276, S281 (see Figure 5.1.), implying the mandatory character of these residues in regulating NF-κB transcriptional activity. On the other hand, several RelA residues undergoing post-translational modifications, i.e. K37, K310, S311, K314, K315 (see Figure 5.1.), seem to be used by evolution as a novelty, possibly enhancing the complexity of NF-κB transcriptional regulation. Finally, several RelA S, T and Y residues are highly conserved, e.g. Y36, S42, S51, T57, T78, S180, Y257 (see Figure 5.1.), but have not been described to undergo post-translational modifications or to influence NF-κB transcriptional activity. Thus, understanding the sequence of and the interplay between different post-translational modifications could be of importance in developing therapeutic strategies that effectively target a specific NF-κB post-translational modification and hence the expression of genes regulated

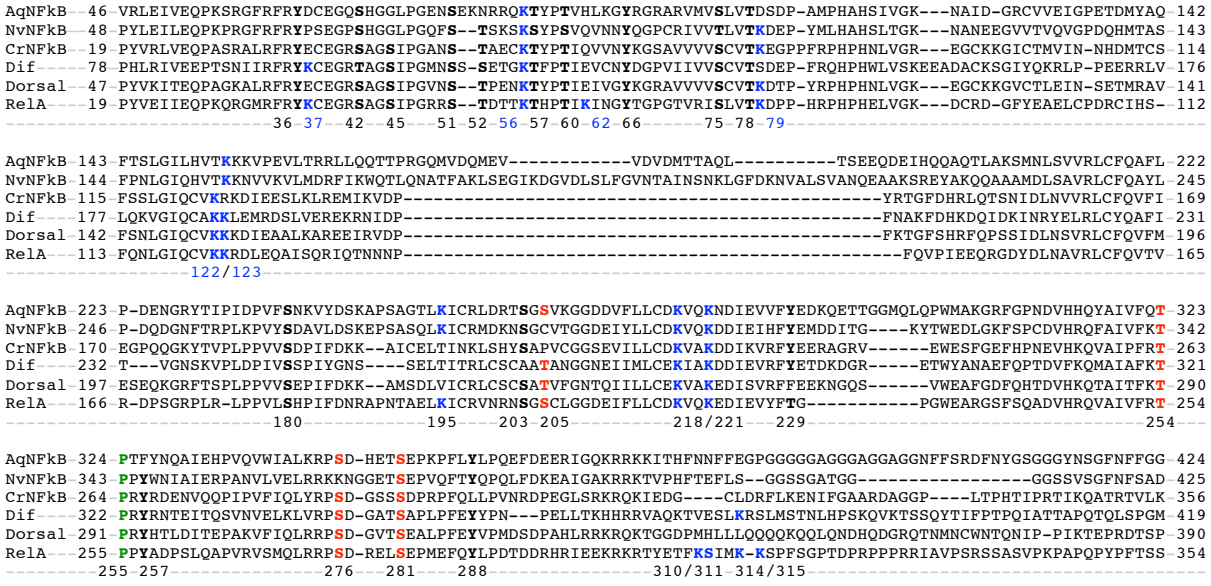

Figure 5.1. Protein sequence alignment of different Rel proteins. Comparative analysis of NF-κB RHD among different speciaes, i.e. *Amphimedon queenslandica* (Aq), *Nematostella vectensis* (Nv), *Carcinoscorpius rotundicauda* (Cr), *Drosophila melanogaster* (Dif, Dorsal) and *Homo sapiens* (RelA) shows high conservation of several RelA serine (S), threonine (T) and tyrosine (Y) residues undergoing phosphorylation (red), lysine (K) residues undergoing acetylation, methylation and/or ubiquitilation (blue) and S, T, Y and K residues not shown to undergo PTMs (bold black).

by that particular post-translational modification. It should be noted, however, that If post-translational modifications happen as a sequence of events, early upstream post-translational modification should not only regulate the transcription of subset of NF-κB-dependent genes but also induce or inhibit another post-translational modification and hence indirectly affect a different subset of genes regulated by that other post-translational modification. As an example, RelA S276 phosphorylation can promote the binding of RelA to CBP/p300, which, in addition to acting as a transcriptional activator of NF-κB-dependent gene transcription, acetylates RelA at K310 (187). Moreover, K310 acetylation inhibits RelA K314 and K315 methylation and thus methylation-induced RelA ubiquitination and consequent degradation (204, 374). As such, modulating HO-1 activity and hence RelA S276 phosphorylation would likely affect broad range of genes whose transcription depends on both histone and RelA acetylation. On the other hand, modulating CCT activity and hence RelA acetylation would rather regulate only a specific subset of NF-κB dependent genes. Further understanding of the kinetics and the cross-talk between different NF-κB post-translational modifications over the course of inflammatory response is therefore required to provide new targets and strategies for antimicrobial therapies.

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